University of Alberta

Alternative Splicing of *Disabled-1* in the Developing Retina

by

Sachin Katyal ©

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of the requirements for the degree of Doctor of Philosophy

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For my grand-parents, the late Mr. Jagan Nath Katyal, Mrs. Gian Vanti Katyal, the late Mr. Chaman Lal Malhotra and the late Mrs. Parkash Malhotra.

ABSTRACT

The Reelin-Disabled 1 (Dab1) signaling pathway is implicated in the positioning of migrating neurons in the brain. Reelin is a secreted extracellular matrix glycoprotein that binds to two types of receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2). Reelin signaling activates the intracellular adaptor protein Dab1 through phosphorylation of tyrosine(Y)-198 which in turn stimulates the phosphorylation and activation of Src-family kinases (SFKs). SFKs are involved in Dab1 phosphorylation while phosphorylated Dab1 enhances their activity, suggesting a self-regulating tyrosine signaling cascade. Activated Dab1 modulates the phosphorylation of a variety of downstream effectors involved in the regulation of cell morphology and neuronal cell migration.

Using RT-PCR analysis, we have isolated two alternatively spliced isoforms of Dab1 from human and chick retina: Dab1-E(early) and Dab1-L(late). Dab1-L contains four tyrosines found within two SFK (Y185 and Y198) and two Abl kinase/Crk (Y220 and Y232) recognition motifs, while Dab1-E only has the Abl/Crk motifs. We demonstrate that expression of these two isoforms is spatio-temporally regulated by a timed developmental stage-specific alternative splicing event. Based on *in situ* hybridization and fluorescence immunolocalization analyses of retinal tissue sections, we find that Dab1-E is expressed in undifferentiated retinal precursor cells while Dab1-L expression is restricted to amacrine and ganglion cells. Transfection of primary chick retinal cultures with Dab1-E and Dab1-L expression constructs reveals specific roles for these two

isoforms, with Dab1-L-expressing cells characterized by increased phosphotyrosine levels, activated SFKs and the formation of thin neurite-like processes, while Dab1-E-expressing cells show reduced phosphotyrosine levels, no SFK activity and retain an undifferentiated epithelial-like morphology. Transfection of Dab1-L expression constructs mutated singly, doubly or triply at Y185, Y198, Y220 and Y232 reveal a hierarchical relationship between these four tyrosines, with tyrosine-198 being essential for Reelin-mediated Dab1 tyrosine phosphorylation, and tyrosine-232, combined with either tyrosine-185 or tyrosine-220, being required for full Dab1^{Y198}-mediated SFK activation and neurite formation.

We have also examined Dab1 expression in two tumours of neuroectodermal cell origin, retinoblastoma and neuroblastoma. We have identified five different *Dab1* splice variants in both cell lines and tumour tissues, suggesting that *Dab1* alternative splicing is deregulated in these tumours. Furthermore, western blot analysis indicates that Reelin and Dab1 protein are either not expressed or barely detectable in tumour cell lines suggesting that the Reelin-Dab1 signaling pathway may need to be inactivated during tumorigenesis.

We propose that Dab1 alternative splicing acts as a Reelin-Dab1 uncoupling-recoupling signaling mechanism to modulate neuronal proliferation and differentiation signals within a Reelin-positive environment. These findings reveal a novel signal transduction paradigm involving alternative splicing to modulate intracellular responses to extracellular stimuli.

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LIST OF ABBREVIATIONS

Abbreviation	**Full name**
%	percent
2-D	two dimensional
aa	amino acid
Abl	Abelson
AD	activating domain
ade	adenine
AP-2	adaptor protein 2
AP-2β	activating-protein 2 beta
APLP1	amyloid precursor-like protein 1
APLP2	amyloid precursor-like protein 2
ApoER2	apolipoprotein E receptor 2
APP	amyloid precursor protein
ARH	autosomal recessive hypercholesterolemia
Arp2/3	actin-related protein 2/3
ASF/SF2	alternative splicing factor/splicing factor 2
Asn	asparagine
Azi1	5-azacytidine induced gene-1
Bcl-x(L)	B-cell CLL/lymphoma-related protein long isoform
Bcl-x(s)	B-cell CLL/lymphoma-related protein short isoform
BD	binding domain
bHLH	basic-helix-loop-helix
bp	base pair
C-terminus	carboxy terminus
C3G	guanine nucleotide-releasing protein
Ca^{2+}	cationic calcium
CA-II	carbonic anhydrase-II
Cbl	Cas-BR-M murine ecotropic retroviral transforming sequence homologue
Cdc42	cell division cycle 42
Cdk5	cyclin-dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
CFTR	cystic fibrosis transmembrane conductance regulator
ch	chick
Chx10	ceh10 homeodomain-containing homologue
CR	Cajal-Retzius
CRIB	Cdc42/Rac-interactive binding
CrkL	Crk-like
Crx	cone-rod homeobox-containing gene
D	aspartic acid/aspartate
Dab1	Disabled-1
Dab1-E	Disabled-1 early isoform

Dab1-L	Disabled-1 late isoform
Dab2	Disabled-2
Dab2IP	Disabled-2-interacting protein
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DD-PCR	differential display polymerase chain reaction
DEL	deletion region
DI	diencephalon
DIG	digoxigenin
DNA	deoxyribonucleic acid
DOC-2	differentially expressed in ovarian cancer 2
DOCK	dedicator of cytokinesis
DTT	dithiothreitol
E	glutamic acid/glutamate
E	embryonic day (mouse)
ED	embryonic day (chick)
EGF	epidermal growth factor
ESE	exonic splicing enhancer
ESS	exonic splicing suppressor
FGFR2	fibroblast growth factor receptor 2
F	phenylalanine
Foxn4	Forkhead box N4
GAP-43	growth associated protein 43
GCL	ganglion cell layer
GFAP	glial fibrillary acid protein
GFP	green fluorescent protein
Gln	glutamine
Glu	glutamic acid/glutamate
GluR2	glutamate receptor-2 subunit
GSK-3β	glycogen synthase kinase 3 beta
Hes1	hairy/enhancer of split, Drosophila, homologue of, 1
Hes5	hairy/enhancer of split, Drosophila, homologue of, 5
Hesr2	hairy/enhancer of split, Drosophila, homologue of, related 2
his	histidine
hnRNP	heterogeneous nuclear ribonucleoprotein
hr	hour
hu	human
g	gram
I	isoleucine
INBL	inner neuroblastic layer
INL	inner nuclear layer
INS	insertion region
Ins1,4,5P_3	inositol-1,4,5-triphosphate
IPL	inner plexiform layer
ISE	intronic splicing enhancer
Iso	isoleucine

ISS	intronic splicing suppressor
kDa	kilodalton
KIF3B	kinesin family member 3B
LDLR	low density lipoprotein receptor
leu	leucine
LGN	lateral geniculate nucleus
LIS1	lissencephaly 1
LP	lens placode
LRP	lipoprotein receptor-related protein
LV	lens vesicle
m	adenine or cytidine
M	molar
MAPK	mitogen activated protein kinase
Mash1	mammalian achaete-scute homologue 1
MEFs	mouse embryonic fibroblasts
µg	microgram
mg	milligram
Mitf	micropthalmia-associted transcription factor
µL	microlitre
ml	millilitre
µm	micrometer
mm	millimeter
µM	micromolar
mM	millimolar
mRNA	messenger ribonucleic acid
n	any nucleotide
N	asparagine
N-terminus	amino terminus
NB	neuroblastoma
Nckα	Nck adaptor protein alpha
Nckβ	Nck adaptor protein beta
NeuroD	neurogenic differentiation gene
NFB42	neuronal F-box protein 42
NFL	nerve fibre layer
NICD	Notch intracellular domain
NR	neural retina
nt	nucleotide
NTK	non-receptor tyrosine kinase
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
OC	optic cup
-OH	hydroxyl group
OS	optic stalk
Otx2	orthodenticle, Drosophila, homologue of, 2
OV	optic vesicle
ONBL	outer neuroblastic layer
ONL	outer nuclear layer

OPL	outer plexiform layer
P	postnatal
P	proline
P1	primer 1
p130(CAS)	Crk-associated substrate 130
p-tyr	phosphotyrosine
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1B, alpha subunit
Pax6	paired box gene 6
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PH	pleckstrin homology
Phe	phenylalanine
PI/PTB	protein interaction/phosphotyrosine-binding domain
PI3K	phosphoinositol 3' kinase
PKC	protein kinase C
PLCγ1	phospholipase C-gamma1
Pol II	RNA polymerase II
Pro	proline
Prox1	prospero-related homeobox 1
p-Src	phosphoSrc
pSFK	phospho-Src family kinase
PTEN	phosphatase and tensin homologue
pTyr	phosphotyrosine
Pi	inorganic phosphate
poly(A)$^+$	poly-adenylated
PRL-2	phosphatase of regenerating liver 2
PtdIns3,4P$_2$	phosphoinositol-3,4-bisphosphate
PtdIns3,4,5P$_3$	phosphoinositol-3,4,5-triphosphate
PtdIns4P	phosphoinositol-4-phosphate
PtdIns4,5P$_2$	phosphoinositol-4,5-bisphosphate
Q	glutamine
Rac	Ras-related C3 botulinum toxin substrate
RAP	receptor-associated protein
RAP1	Ras-related protein 1
Rax	Retina and anterior neural fold gene
RET	rearrangement during transfection proto-oncogene
RGC	retinal ganglion cell
RNA	ribonucleic acid
RPC	retinal progenitor cell
RB	retinoblastoma
RPE	retinal pigmented epithelium
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-mediated polymerase chain reaction
S	serine
SDS	sodium dodecyl sulfate

SE	surface ectoderm
SF1	splicing factor 1
SFK	Src-family kinase
SGT1	suppressor of G2 allele of SKP1
SH2	Src-homology 2 domain
SH3	Src-homology 3 domain
Shh	sonic hedgehog
SHIP	SH2-containing inositol phosphatase
SHP-2	SH2-containing protein tyrosine phosphatase 2
Six3	sine oculis homeobox, Drosophila, homologue of, 3
Six6	sine oculis homeobox, Drosophila, homologue of, 6
siRNA	small interfering ribonucleic acid
snRNP	small nuclear ribonucleoprotein
SP	F-spondin
SR	serine-arginine protein
SSC	sodium chloride-sodium citrate
sub a	sublamina a (OFF)
sub b	sublamina b (ON)
SUV420H	suppressor of variegation 420 homologue
T	threonine
TBS	Tris-buffered saline
Thr	threonine
tRNA	transfer ribonucleic acid
trp	tryptophan
Tyr	tyrosine
u	purine-based nucleotide
U2AF35	U2 auxiliary factor 35 kDa-subunit
U2AF65	U2 auxiliary factor 65 kDa-subunit
UTR	untranslated region
V	valine
Val	valine
VLDLR	very low density lipoprotein receptor
wk	week
X	any amino acid
y	pyrimidine-based nucleotide
Y	tyrosine
YFP	yellow fluorescent protein

CHAPTER 1

Introduction

1.1 EYE AND RETINAL DEVELOPMENT

Formation of the vertebrate retina involves a complex interplay of cell-extrinsic factors and cell-intrinsic events (Edlund and Jessell, 1999). The overexpression or inactivation of genes in a variety of animal model systems has helped us to delineate key pathways involved in retinal development and specification of retinal cell lineages (Inoue *et al.*, 1990; Marquardt and Gruss, 2002). However, in order to understand the full spectrum of molecular events that govern retinal development, we will need to identify all the biochemical factors, their targets and their interplay in the variety of pathways involved in retinal formation and differentiation. A confounding factor is that the expression of a specific molecule may have different consequences depending on: (i) when it is expressed during retinogenesis, (ii) which cell types it is expressed in, and (iii) what other molecules are co-expressed with it. An important goal is therefore to understand the delicate balance between factors that affect proliferation of retinal cells and their differentiation along specific cell lineages.

1.1.1 Vertebrate Eye Formation

One of the earliest events in the formation of the vertebrate eye is the specification of the eye field in the anterior neural plate of the neural tube (Oster *et al.*, 2004). The optic vesicle (OV) forms from the eye field as a lateral evagination from the walls of the embryonic diencephalon (DI), a region of the neural tube destined to become the forebrain (Fig. 1.1A) (Walls, 1963; Grun, 1982; Poley *et al.*, 1989). The optic vesicle remains attached to the presumptive

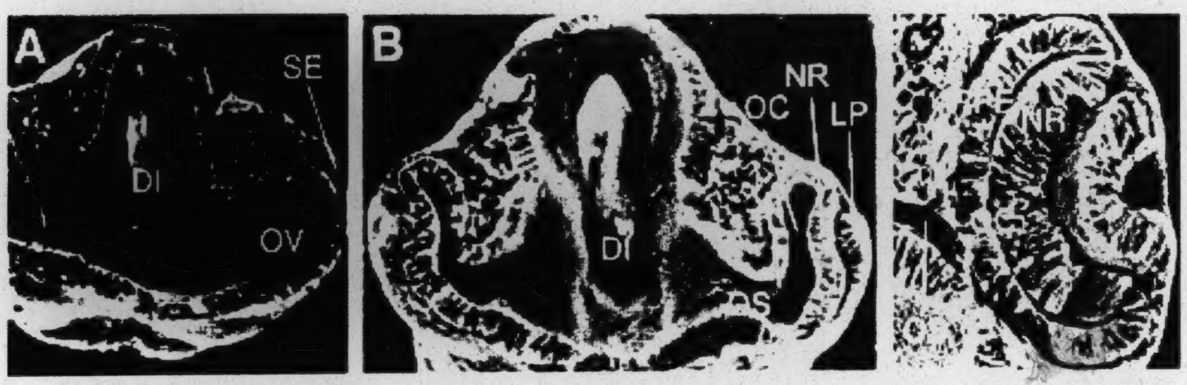


Fig. 1.1 - Vertebrate eye development. Abbreviations are as follows: SE, surface ectoderm; DI, diencephalon; OV, optic vesicle; OS, optic stalk; OC, optic cup; NR, neural retina; LP, lens placode; RPE, retinal pigmented epithelium; LV, lens vesicle.

Fig. 1.1 modified from http://www.mpibpc.gwdg.de/inform/MpiNews/cientif/jahrg7/7.01/scta.html forebrain via the optic stalk (OS) (Figs. 1.1B, 1.1C). Contact between the optic vesicle and the overlying ectoderm results in the thickening of a portion of the surface ectoderm (SE) (Fig. 1.1A), a region destined to form the lens placode (LP) (Figure 1B) (Romanoff, 1960). The lens placode evaginates and detaches from the surface ectoderm to form the lens vesicle (LV), the presumptive lens (Fig. 1.1C). During lens formation, the optic fissure forms as the result of an invagination of the ventral aspect of the optic vesicle and optic stalk. Fusion of tissue derived from the two opposing ends of the optic fissure gives rise to the double-layered optic cup (OC) (Figs. 1.1B, 1.1C). The outer layer differentiates into the iris, ciliary body and the retinal pigmented epithelium (RPE) while the inner layer differentiates into the neural retina (NR), which lines the back of the eye, adjacent to the RPE (Figs. 1.1C, 1.2) (Sivak and Sivak, 2000; Chow and Lang, 2001)

1.1.2 Retinal development

The retina is composed of specialized cell types derived from a common neuroectodermal progenitor cell, referred to as retinal progenitor cell (RPC). RPCs have the potential to differentiate into six neuronal cell types (photoreceptor, horizontal, bipolar, interplexiform, amacrine and ganglion) and one glial cell type (Müller) (Fig. 1.2B) (Farber and Adler, 1986; Dowling, 1987; Turner and Cepko, 1987; Wetts and Fraser, 1988; Wetts *et al.*, 1989; Turner *et al.*, 1990; Cepko *et al.*, 1995). The structure of the neural retina is well-conserved among all vertebrates. As such, conserved signaling pathways

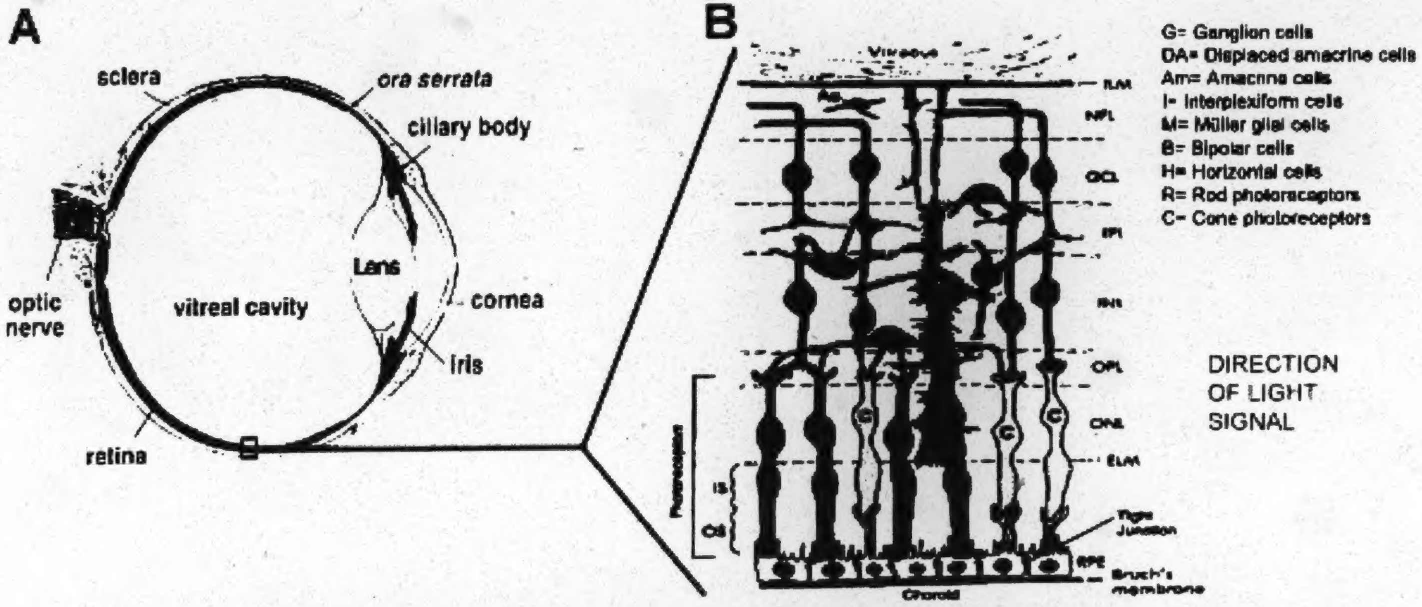

A
sclera
ora serrata
ciliary body
optic nerve
vitreal cavity
Lens
cornea
iris
retina
B
Vitreous
ILM
Am
NFL
GCL
IPL
INL
OPL
ONL
ELM
C
Photoreceptors
IS
OS
Tight Junction
RPE
Bruch's membrane
Choroid
G= Ganglion cells
DA= Displaced amacrine cells
Am= Amacrine cells
I= Interplexiform cells
M= Müller glial cells
B= Bipolar cells
H= Horizontal cells
R= Rod photoreceptors
C= Cone photoreceptors
DIRECTION OF LIGHT SIGNAL

Fig. 1.2 - Schematic of vertebrate eye (A) and retinal organization (B). (A) The retina (in red) lines the back of the eye. Light enters through the lens and is captured by photoreceptor cells (C and R in B) of the outer nuclear layer (ONL). This signal is sent through the outer plexiform layer (OPL) where cells of the inner nuclear layer (INL) process the visual signal. This signal is then transduced through the inner plexiform layer (IPL) to the ganglion cell layer (GCL) whereby it is projected onto the brain via the optic nerve.

Fig. 1.2A modified from http://www.mpibpc.gwdg.de/inform/MpiNews/cientif/jahrg7/7.01/scta.html; Fig. 1.2B modified from Ogden, 1987 mediate the specific temporal pattern of retinal cell specification. This specification is complex as it has to occur in an environment where cell proliferation, cell commitment and cell differentiation are all happening simultaneously (Fig. 1.3A) (Levine and Green, 2004). RPC division can result in the formation of new RPCs as well as cells committed to a specific lineage (Sidman, 1961; Yang, 2004). Retinal progenitor cell nuclei undergo cell-cycle-dependent movement and the location of the nuclei during cell division determines the final positioning of differentiating neurons. As a result, upon exit from the cell cycle, post-mitotic neurons localize close to their final laminar position within the ventricular zone while proliferating cells are found at the ventricular surface. Numb is a cell-fate determining protein shown to alter cell polarity and the plane of daughter cell cleavage during RPC cell division (Cayouette and Raff, 2003; Dooley *et al.*, 2003). Symmetrical localization of Numb in the dividing RPC results in horizontal cell division and produces two daughter cells with similar progenitor potency as the parental cell. Asymmetrical localization of Numb results in vertical cell division with one daughter cell remaining at the ventricular surface to continue as an RPC, while the other occupies the ventricular zone to differentiate along a particular retinal lineage. Early cell divisions specify early post-mitotic retinal cell types while later cell divisions specify late post-mitotic cell types. Therefore, RPCs must balance two opposing forces: (i) proliferation at an appropriate rate to accumulate enough cells to generate late retinal cell types and, (ii) timely cell cycle exit to form early-born retinal cell types.

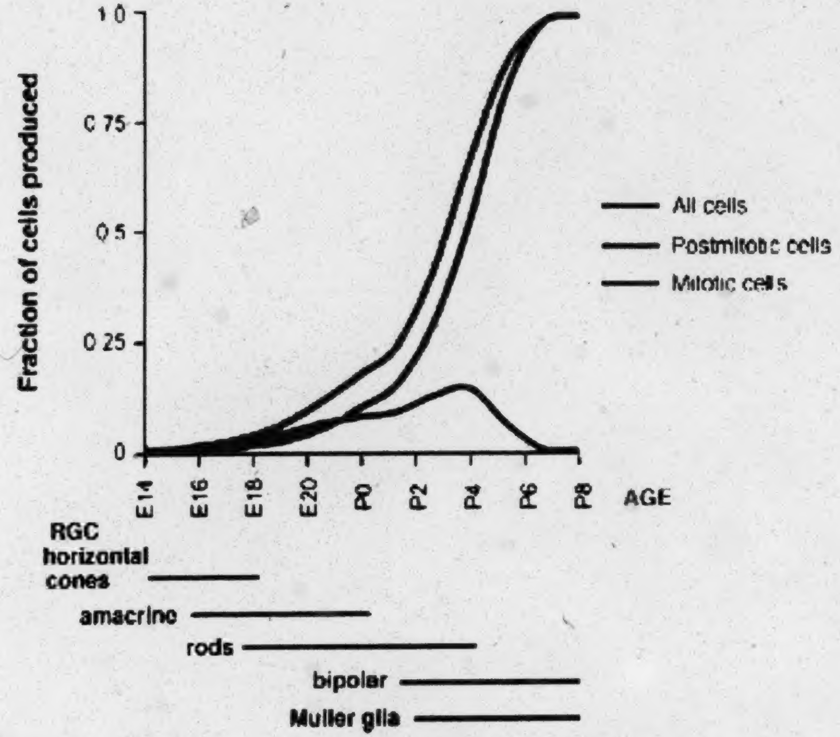
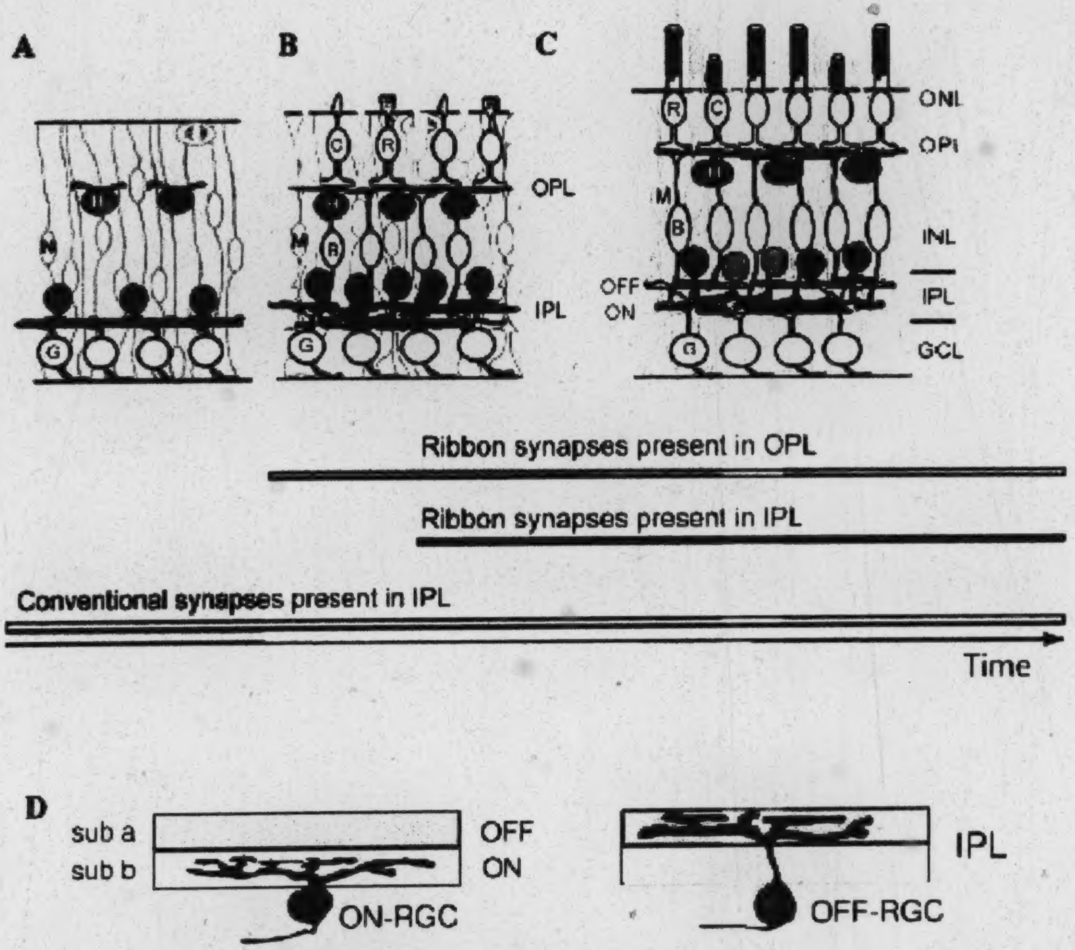


Fig. 1.3 - Number and spatiotemporal appearance of retinal cell types in mouse

Fig. 1.3 modified from Levine and Green, 2004

The earliest cell type to form in the retina is ganglion (Fig. 1.3), which appears upon closure of the optic fissure (Oster *et al.*, 2004). The next cell types to appear are amacrine, horizontal and cone photoreceptor, followed by rod photoreceptor, bipolar and then Müller glia (Romanoff, 1960; Boycott and Dowling, 1969; Prada *et al.*, 1991; Cepko *et al.*, 1996). The concerted appearance of these cell types allows their positioning into a well-organized laminated structure composed of three cell layers interconnected by two synaptic layers (Fig. 1.2B). Light photons are captured by rod and cone photoreceptors of the outer nuclear layer (ONL) which transmit the neural signal across the outer plexiform layer (OPL) to the inner nuclear layer (INL). Horizontal, interplexiform, bipolar and amacrine cells process and transmit the signal through the inner plexiform layer (IPL) to the ganglion cell layer (GCL) where ganglion cell axons in the nerve fiber layer carry the visual signal through the optic nerve to the visual cortex (Dowling, 1987).

The formation of the mature retinal signaling network, linking the INL to the ONL and GCL, occurs in a stepwise fashion (Tian, 2004; Mumm *et al.*, 2005). Conventional synapses first occur through interneuron connections at the IPL between amacrine and ganglion cells (Fig. 1.4A) followed by connections at the OPL between photoreceptor and horizontal cells (Fig. 1.4B). Bipolar cells form ribbon synapses with photoreceptor cells at the OPL and with retinal ganglion cells (RGCs) at the IPL, thereby linking the photoreceptor-horizontal cell network to the amacrine-ganglion cell network (Figs. 1.4B, 1.4C). Bipolar, amacrine and ganglion cell dendrites form arbors which stratify into two distinct sublaminae



A
B
C
ONL
OPL
INL
IPL
GCL
OFF
ON
G
R
C
M
B
Ribbon synapses present in OPL
Ribbon synapses present in IPL
Conventional synapses present in IPL
Time
D
sub a
sub b
OFF
ON
ON-RGC
IPL
OFF-RGC

Fig. 1.4 - Formation of retinal plexiform layers during retinal maturation

Fig. 1.4 modified from Mumm et. al., 2005 within the IPL: ON (sublamina b) and OFF (sublamina a) (Figure 1.4C). These sublaminae specify parallel ON and OFF pathways which act to separate excitory and inhibitory visual inputs. ON-bipolar cells connect with ON-RGCs at sublamina b, while OFF-bipolar cells connect with OFF-RGCs at sublamina a (Fig. 1.4D). However, not all bipolar cell processes synapse directly with ganglion cell arbors, as amacrine cells specify the rod-driven system while bipolar cells specify the cone-driven system. Whereas cone-driven bipolar cells synapse vertically with RGCs, amacrine cells derive rod-driven signals from rod-bipolar cells and transmit this signal laterally within the IPL to RGC dendrites. The signal is then transmitted to the brain via RGC axons.

1.1.3 Molecular specification of eye formation

A seemingly endless number of transcription factors, signaling molecules and other biochemical factors are involved in eye and retinal development. These proteins have multiple roles intersecting with a variety of pathways. Due to the enormous volume of literature on this topic, only a few key factors involved in both eye and retinal development will be described here.

Many factors that specify retinal cell lineages are initially expressed early in eye morphogenesis (Fig. 1.5A). As the retina starts to differentiate after optic cup formation, the expression pattern of these factors narrows to specific retinal cell lineages. Homeobox genes such as *Rx/Rax, Pax6, Six3* and *Six6/Optx2* play important roles in both eye formation and retinal development (Casarosa *et al.*, 1997; Furukawa *et al.*, 1997; Mathers *et al.*, 1997; Toy *et al.*, 1998; Jean *et*

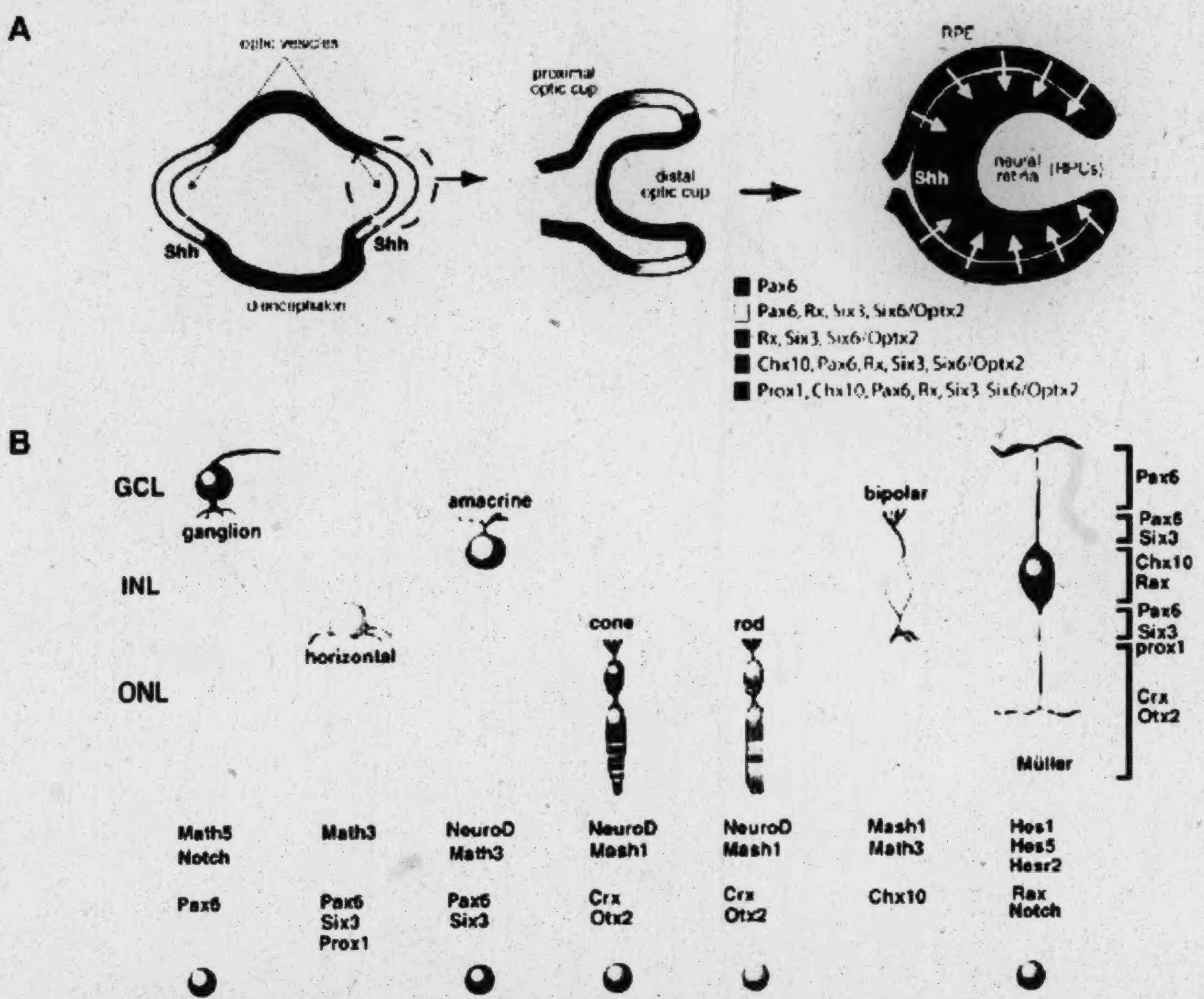

A
optic vesicles
Shh
Shh
diencephalon
proximal optic cup
distal optic cup
RPE
Shh
neural retina (RPCs)
■ Pax6
☐ Pax6, Rx, Six3, Six6/Optx2
■ Rx, Six3, Six6/Optx2
■ Chx10, Pax6, Rx, Six3, Six6/Optx2
■ Prox1, Chx10, Pax6, Rx, Six3, Six6/Optx2
B
GCL
INL
ONL
ganglion
horizontal
amacrine
cone
rod
bipolar
Müller
Pax6
Pax6
Six3
Chx10
Rax
Pax6
Six3
prox1
Crx
Otx2
Math5
Notch
Pax6
Math3
Pax6
Six3
Prox1
NeuroD
Math3
Pax6
Six3
NeuroD
Mash1
Crx
Otx2
NeuroD
Mash1
Crx
Otx2
Mash1
Math3
Chx10
Hes1
Hes5
Hesr2
Rax
Notch

Fig. 1.5 - Genes involved in the specification of vertebrate eye development (A) and formation of retinal subtypes (B)

Fig. 1.5A modified from Levine and Green, 2004; Fig. 1.5B modified from Hatakeyama and Kageyama, 2004 *al.*, 1999; Lopez-Rios *et al.*, 1999; Toy and Sundin, 1999; Kawakami *et al.*, 2000; Zhou *et al.*, 2000; Ghanbari *et al.*, 2001). Specification of the eye field is mediated by Rx/Rax expression while induction of *Pax6* initiates the lateral evagination event required for optic vesicle formation (Grindley *et al.*, 1995; Chow and Lang, 2001; Levine and Green, 2004). Initially, both layers of the optic cup (the presumptive neural retina and RPE) express Pax6; however, final specification of the RPE requires repression of *Pax6* and expression of the basic helix-loop-helix (bHLH) factor *Mitf* and homeodomain gene *Otx2* (Walther and Gruss, 1991; Mochii *et al.*, 1998; Martinez-Morales *et al.*, 2001). Formation of the inner layer of the optic cup, the presumptive neural retina, is mediated by expression of Chx10, Pax6, Rx, Six3, Six6/Optx2 and many other factors (Liu *et al.*, 1994; Levine *et al.*, 1997).

1.1.4 Molecular cues regulating RPC proliferation and multipotency

One of most important genes responsible for specifying RPC mutipotency is *Pax6*, often considered a master regulator of retinal development. Conditional inactivation of *Pax6* in the inner layer (presumptive retina) of the optic cup prior to the onset of retinal neurogenesis results in a failure to produce any of the cell lineages except for amacrine (Marquardt *et al.*, 2001). Overexpression of Pax6 promotes the proliferation of RPCs, However, when Pax6 is over-expressed along with Math3 and NeuroD or Crx (discussed below), it promotes the differentiation of non-photoreceptor neurons and inhibits differentiation of photoreceptor neurons (Inoue *et al.*, 2002; Toy *et al.*, 2002). Similarly, another homeodomain gene, *Chx10*, is also implicated in non-photoreceptor neuron differentiation and in the promotion of INL cell number (Hatakeyama *et al.*, 2001; Toy *et al.*, 2002; Green *et al.*, 2003). *Ocular retardation* mice (*Chx10*$^{-/-}$ mutant mice) display early retinal hypoplasia and severely reduced retinal cell numbers by birth. This phenotype is attributed to significantly longer RPC cell-cycling times, resulting in reduced rates of progenitor cell proliferation (Burmeister *et al.*, 1996). Secretion of cell-extrinsic Shh by the adjacent RPE (Fig. 1.5A) also acts to increase RPC proliferation (Levine *et al.*, 1997; Stenkamp *et al.*, 2000). Cell-extrinsic Notch signaling activates the cell-intrinsic expression of the bHLH transcriptional repressor Hes1, a molecular sensor for progenitor cell accumulation (Ohtsuka *et al.*, 1999; Davis and Turner, 2001). When an appropriate number of RPCs accumulate, *Hes1* is turned off to allow retinal cell specification. Misexpression of Hes1 inhibits retinal differentiation, while *Hes1* inactivation in the embryonal retina causes the small-eye phenotype resulting from premature differentiation of too few progenitor cells (Ishibashi *et al.*, 1995; Tomita *et al.*, 1996a). Similarly, the homeobox gene *Prox1* is implicated in progenitor cell proliferation and cell-fate specification. Cells lacking functional Prox1 have reduced capacity to exit the cell cycle, while Prox1 overexpression forces premature cell-cycle exit (Dyer *et al.*, 2003).

1.1.5 Molecular specification of retinal cell lineages (summarized in Fig. 1.5B)

RGC specification involves a number of genes which function in a hierarchical manner, the most important of which include *Notch, Pax6* and *Ath5*.

Notch, a cell surface receptor, is in fact a negative regulator of RGC production (Austin *et al.*, 1995; Dorsky *et al.*, 1995). Notch-ligand binding, by cell-extrinsic factors Delta and/or Serrate, induces a proteolytic cleavage resulting in the nuclear translocation of the Notch intracellular domain (NICD) (Baron, 2003; Selkoe and Kopan, 2003). The NICD functions as a transcriptional co-activator with the DNA-binding CSL transcription factor to activate the bHLH transcriptional repressors *Hes1* and *Hes5* (discussed below) (Ohtsuka *et al.*, 1999). However, the NCID also acts to inhibit Ath5 expression, likely through direct transcriptional repression or indirectly via Hes1/Hes5-mediated repression (Schneider *et al.*, 2001). Conversely, Pax6 is an activator of Ath5 (Marquardt *et al.*, 2001). Therefore, it is postulated that the opposing functions of Pax6 and Notch somehow ensure precise expression of Ath5 (Mu and Klein, 2004). *Ath5* inactivation studies demonstrate a complete loss of ganglion cells and over-production of amacrine cells, while Ath5 over-expression increases ganglion cell number at the expense of amacrine and bipolar cells (Kanekar *et al.*, 1997; Brown *et al.*, 2001; Kay *et al.*, 2001; Wang *et al.*, 2001b). Therefore, Pax6-mediated activation and regulation of *Ath5* is absolutely necessary in conferring RPC competence to the ganglion cell lineage.

Two genes critical to amacrine cell specification are the bHLH transcription factors *Ath3* and *NeuroD*. Both these genes are transiently expressed during amacrine cell differentiation. Inactivation of *NeuroD* results in delayed amacrine cell production, while inactivation of *Math3* (murine *Ath3)* has no effect on amacrine cell development (Morrow *et al.*, 1999; Tomita *et al.*, 2000). Interestingly, *Math3*$^{-/-}$ *NeuroD*$^{-/-}$ double-mutant mice demonstrate a severe reduction in amacrine cells accompanied by an increase in retinal ganglion cells, likely attributed to an upregulation of Ath5 expression (Inoue *et al.*, 2002). This suggests that both Math3 and NeuroD share functional redundancy, yet when combined, they have important functions in promoting amacrine cell lineage formation and suppressing ganglion cell specification by antagonizing Ath5 signaling. Although *Math3*$^{-/-}$ *NeuroD*$^{-/-}$ double-mutant mice display reduced amacrine cell numbers, formation of some amacrine cells is observed. Furthermore, the mild phenotype in *NeuroD*$^{-/-}$ mutant mice, the lack of phenotype in *Math3*$^{-/-}$ mutant mice and the transient nature of Math3 and NeuroD expression during amacrine cell production suggests that additional upstream genes are involved in amacrine cell lineage specification. Both the Pax6 and Six3 homeobox proteins are expressed in amacrine cells (Inoue *et al.*, 2002). Although overexpression of either gene individually does not induce amacrine cell production, Pax6 or Six3 co-expression with either Math3 or NeuroD dramatically increases amacrine cell numbers. One interesting observation was noted in the Pax6-Math3-NeuroD co-expression studies: Pax6-NeuroD co-expression resulted in an increase in amacrine cell numbers exclusively while Pax6-Math3 coexpression generated a proportionately higher number of horizontal cells compared to amacrine cells (Inoue *et al.*, 2002). This difference may be explained by the fact that while NeuroD and Math3 expression is transient in amacrine cells, Math3 expression persists in horizontal cells. This would indicate that Pax6/Six3 and Math3 participate in neuronal subtype specification with Pax6/Six3-Math3-NeuroD coexpression specifying the amacrine cell lineage and Pax6/Six3-Math3 coexpression specifying the horizontal cell lineage (Hatakeyama and Kageyama, 2004). This would also suggest that the molecular mechanisms specifying amacrine and horizontal subtype formation may be closely related.

Math3$^{-/-}$ mutant mice show normal horizontal cell formation, indicating that additional factors must specify the horizontal cell lineage. One such factor is the homeobox gene *Prox1*. While *Prox1* is implicated in early RPC cell-cycle exit events, *Prox1* expression during retinogenesis persists in horizontal cells (Dyer *et al.*, 2003). *Prox1*$^{-/-}$ mutant mice show a complete absence of horizontal cells, implying an essential function in horizontal cell lineage specification. While the exact mechanism by which Prox1 regulates horizontal cell formation is not understood, a recent report indicates that *Prox1*, *Math3* and *NeuroD* are activated by a common upstream regulator: the Foxn4 winged/forkhead transcription factor (Li *et al.*, 2004). Foxn4 expression is associated with proliferating INL neurons (Gouge *et al.*, 2001). *Foxn4*$^{-/-}$ mutant mice display dramatically reduced amacrine cell production and complete loss of horizontal cells, likely attributed to the loss of Prox1 expression and reduced NeuroD and Math3 levels that these mice display (Li *et al.*, 2004).

Specification of the bipolar cell lineage involves *Math3* along with the bHLH factor *Mash1* and the paired-like homeodomain gene *Chx10*. Both *Mash1* and *Math3* are transiently expressed in bipolar cells. *Mash1*-null mice show reduced bipolar cell numbers, while bipolar cell formation is not altered in *Math3*-

null mice (Tomita *et al.*, 2000). Interestingly, bipolar cells do not form in *Mash1*$^{-/-}$ *Math3*$^{-/-}$ double mutant mice, as bipolar-destined neurons undergo gliogenesis and revert to the Müller glial cell lineage. These results indicate that either *Mash1* or *Math3* is required for bipolar cell specification: expression of both genes specifies neuronal (as opposed to glial) cell determination while repression of both genes specifies Müller glial cells. However, the fact that misexpression of either *Mash1* or *Math3* alone does not increase the number of bipolar cells indicates that at least one additional factor is involved in bipolar cell specification (Hatakeyama *et al.*, 2001). *Chx10* is also expressed in bipolar cells and *Chx10*$^{-/-}$ mutant mice display a complete loss of this cell type (Liu *et al.*, 1994; Burmeister *et al.*, 1996). Although the misexpression of *Chx10* alone results in only a general increase in INL cell number, it is the combinatorial misexpression of *Mash1-Chx10* or *Math3-Chx10* that induces bipolar cell production (Hatakeyama *et al.*, 2001). This suggests that all three genes function cooperatively, with *Chx10* mediating layer specification (INL) while *Mash1* and *Math3* specify retinal subtype (bipolar) within this cell layer.

Specification of the light-capturing rod and cone photoreceptor cells of the outer nuclear layer require the joint functions of two homeobox genes, *Otx2* and *Crx*, and the bHLH-containing Mash1 and NeuroD. Both *NeuroD* and *Mash1* are expressed in photoreceptor cells and their inactivation results in a moderate decrease in photoreceptor cells (*NeuroD*-null) and a delay in photoreceptor cell formation (*Mash1*-null), respectively (Ahmad, 1995; Tomita *et al.*, 1996b; Morrow *et al.*, 1999). Although these mutants generate a photoreceptor phenotype, they are not the only factors involved in photoreceptor cell lineage specification. Expression of *Otx2* is essential for photoreceptor cell fate determination and conditional *Otx2*-inactivation results in the conversion of differentiating photoreceptor cells into amacrine-like retinal neurons (Nishida *et al.*, 2003). Furthermore, Otx2 transactivates the Otx-like cone-rod homeobox gene, *Crx*, which is required for terminal differentiation of photoreceptors. As such, inactivation of *Crx* blocks generation of photoreceptor outer segments, leading to a failure to produce the phototransduction apparatus, and severely disrupts synaptic terminal organization within the outer plexiform layer (Morrow *et al.*, 2005). Combined, Mash1, NeuroD, Otx2 and Crx are involved in the stepwise specification and differentiation of photoreceptor cells: Mash1 and Otx2 in initial specification of the photoreceptor cell fate and activation of *Crx* (by Otx2); NeuroD in the final commitment of differentiating photoreceptor cells; and Crx in generating photoreceptor outer segments and OPL synaptogenesis.

Müller glial cells are the only glial cell type to differentiate from RPCs. These cells play critical housekeeping roles including reuptake of synaptic neurotransmitters and the maintainance of correct retinal cell layer lamination (Wang *et al.*, 2002a). Furthermore, Müller glia retain progenitor-like potency for transdifferentiation and repair of neuroretinal damage (Dyer and Cepko, 2000; Fischer and Reh, 2001). Specification of Müller glia involves the homeobox gene *Rx/Rax* and the cell-extrinsic action of the Notch pathway to modulate transcriptional levels of the bHLH transcriptional repressors genes, *Hes1* and *Hes5*, and the *Hes*-related gene, *Hesr2*. All five of these genes have dual functions in proliferation of progenitor cells and induction of gliogenesis, depending on the stage of retinal development. Expression of *Rx/Rax* first occurs during optic vesicle formation, while all fives genes (*Notch, Rx/Rax, Hes1, Hes5, Hesr2*) are expressed in proliferating RPCs. These genes are all down-regulated in mature retinal neurons and their expression becomes restricted to Müller glial cells (Ohtsuka *et al.*, 1999; Furukawa *et al.*, 2000; Hojo *et al.*, 2000). Overexpression of Notch, Rx/Rax or Hes1 in murine RPCs leads to increased expression of Müller glial cell markers, while overexpression of Rx/Rax enhances intrinsic Notch and Hes1 levels (Furukawa *et al.*, 1997; Furukawa *et al.*, 2000). The exact relationship between Rx/Rax-mediated regulation of *Notch* remains poorly understood. *Hes1* and *Hes5* are both essential for Notch-mediated Müller gliogenesis. Notch overexpression in chick retina increases endogenous Hes1 and Hes5 levels, while *Hes1*$^{-/-}$*Hes5*$^{-/-}$ double mutants, as opposed to *Hes1*$^{-/-}$ or *Hes5*$^{-/-}$ single mutants, show loss of Müller glial cells (Ohtsuka *et al.*, 1999). Furthermore, similar to *Hes1* and *Hes5*, *Hesr2* is a primary target of Notch and overexpression of Hesr2 also leads to enhanced gliogenesis (Satow *et al.*, 2001; Iso *et al.*, 2001a). *Hes*-mediated transcriptional repression is thought to occur via Hes factor heterodimerization. Hes1 and Hesr2 have previously been shown to heterodimerize (Iso *et al.*, 2001b). Therefore, it is likely that the gliogenic functions of Hes1, Hes5 and Hesr2 occur through their synergistic activation by Notch and their dimerization to repress neurogenic-specific gene activation.

1.2 SPLICING AND ALTERNATIVE SPLICING

Gene transcription is a process whereby a gene produces a copy of itself in the form of a pre-messenger RNA (mRNA) composed of introns and exons. Specific sequences inherent to exons, introns and exon-intron junctions specify the removal of over 90% of the pre-mRNA through RNA splicing (Stamm *et al.*, 2005). This allows for RNA maturation by shedding extraneous information, typically introns, so that exons may link together to form a continuous protein coding region. On the 5'- and the 3'-ends of the mRNA are the untranslated regions (UTRs). UTRs are regulatory sequences which mediate protein-RNA and RNA-RNA associations thereby affecting RNA activity (Mignone *et al.*, 2002).

The number of known human genes has recently been listed at 19 599, a number that is 10-50 fold less than the number needed to encode the 200 000-1 000 000 proteins required for cellular function (NIH/CEPH Collaborative Mapping Group and et al, 2004). To account for this proteomic diversity, research has focused on the process of alternative splicing of pre-mRNAs. Alternative splicing affects the inclusion and/or exclusion of introns, exons and alternative UTRs to produce alternative mRNAs or isoforms derived from one common gene. These alternatively spliced mRNAs may have subtle or major differences in: (i) the protein coding region thereby altering the sequence, structure and function of the encoded protein, (ii) the regulatory sequences that control their expression, and (iii) RNA secondary structure thereby modulating subcellular RNA localization, translation initiation and RNA abundance (Mignone *et al.*, 2002). It is through alternative splicing that a single gene can account for a multitude of proteins with differing cellular functions. To demonstrate the importance of alternative splicing, a recent study suggests that up to 74% of all human genes have alternatively-spliced forms (Johnson *et al.*, 2003).

1.2.1 Spliceosome assembly and pre-mRNA splicing

The process of pre-mRNA splicing has been well-documented at the molecular level (Black, 2003; Kalnina *et al.*, 2005; Stamm *et al.*, 2005). Key sequence elements specifying spliceosome assembly include the 5' (MAGgu) and 3' (yagG) splice sites, the adenine-containing branchpoint sequence (ynyur$\underline{a}$y), the polypyrimidine tract ($y_{(n)}$), and splicing enhancer and suppressor elements. Small nuclear ribonucleoproteins (snRNPs) and related cofactors bind these splicing regulatory elements which, in turn, associate with each other to form the spliceosome. As illustrated in Fig. 1.6, initial assembly of the spliceosome (E complex) consists of binding of: (i) the U1 snRNP to the 5' splice site (splice donor), (ii) SF1 to the branchpoint sequence, (iii) U2AF65 to the polypyrimidine tract and (iv) U2AF35 to the 3' splice site (splice acceptor). The A complex forms when ATP hydrolysis induces a major structural rearrangement accompanied by replacement of SF1 with U2 snRNP at the branchpoint. Recruitment of the U4/U5/U6 tri-snRNP to U1 and U2 results in another structural rearrangement into the B complex. U6 binding at the 5' splice site results in displacement of U1. The C complex forms when the multi-protein-RNA complex folds, thereby looping out and sequestering the presumptive RNA lariat to be spliced out (ie., an intron). U1 and U4 snRNPs are released from the complex

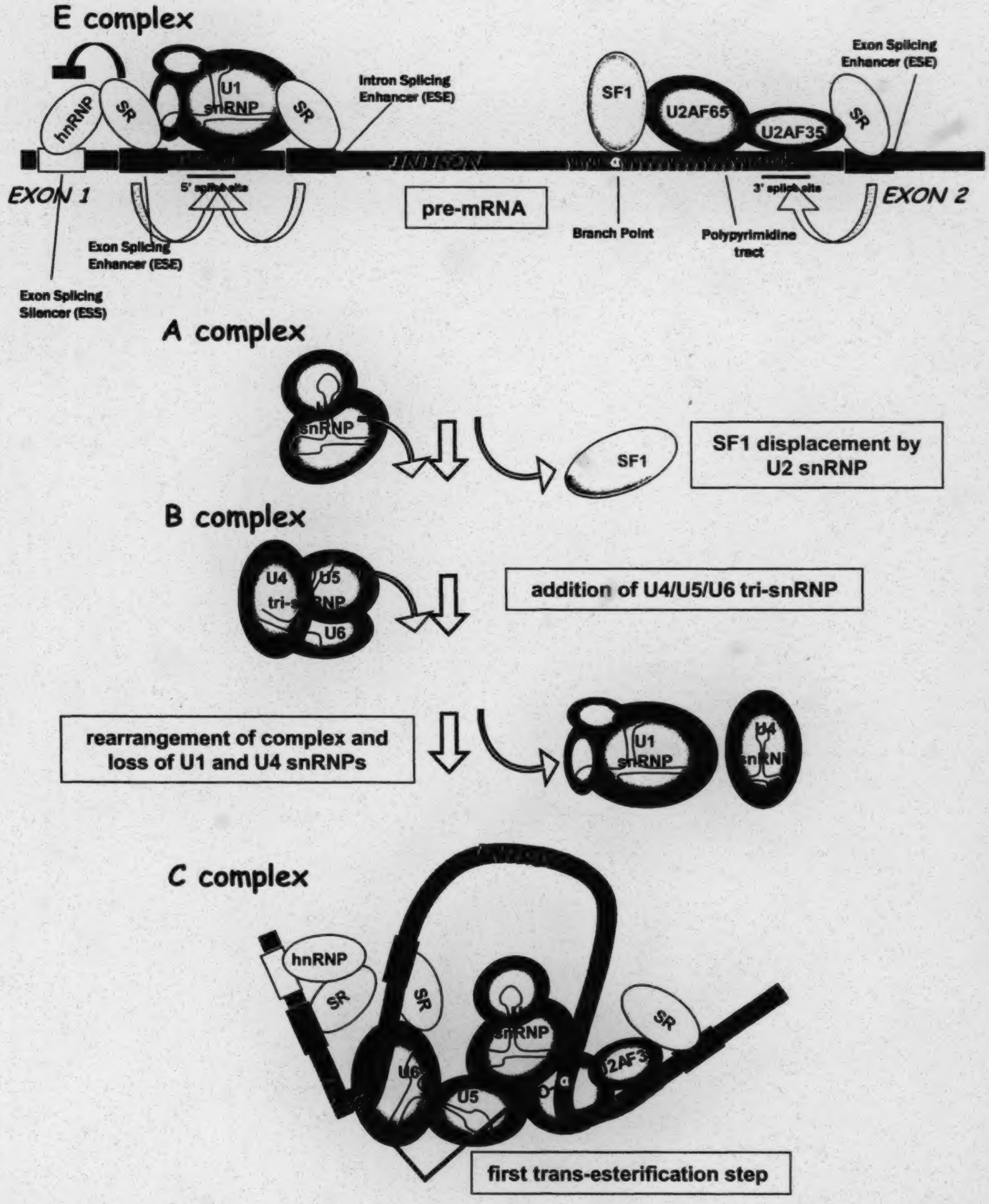

E complex
hnRNP
SR
U1
snRNP
SR
Intron Splicing
Enhancer (ESE)
SF1
U2AF65
U2AF35
SR
Exon Splicing
Enhancer (ESE)
EXON 1
5' splice site
pre-mRNA
Branch Point
Polypyrimidine
tract
3' splice site
EXON 2
Exon Splicing
Enhancer (ESE)
Exon Splicing
Silencer (ESS)
A complex
snRNP
SF1
SF1 displacement by
U2 snRNP
B complex
U4
U5
tri-snRNP
U6
addition of U4/U5/U6 tri-snRNP
rearrangement of complex and
loss of U1 and U4 snRNPs
U1
snRNP
U4
snRNP
C complex
hnRNP
SR
SR
snRNP
SR
U6
U5
U2AF35
first trans-esterification step

Fig. 1.6A - Molecular mechanism of spliceosome assembly and RNA splicing

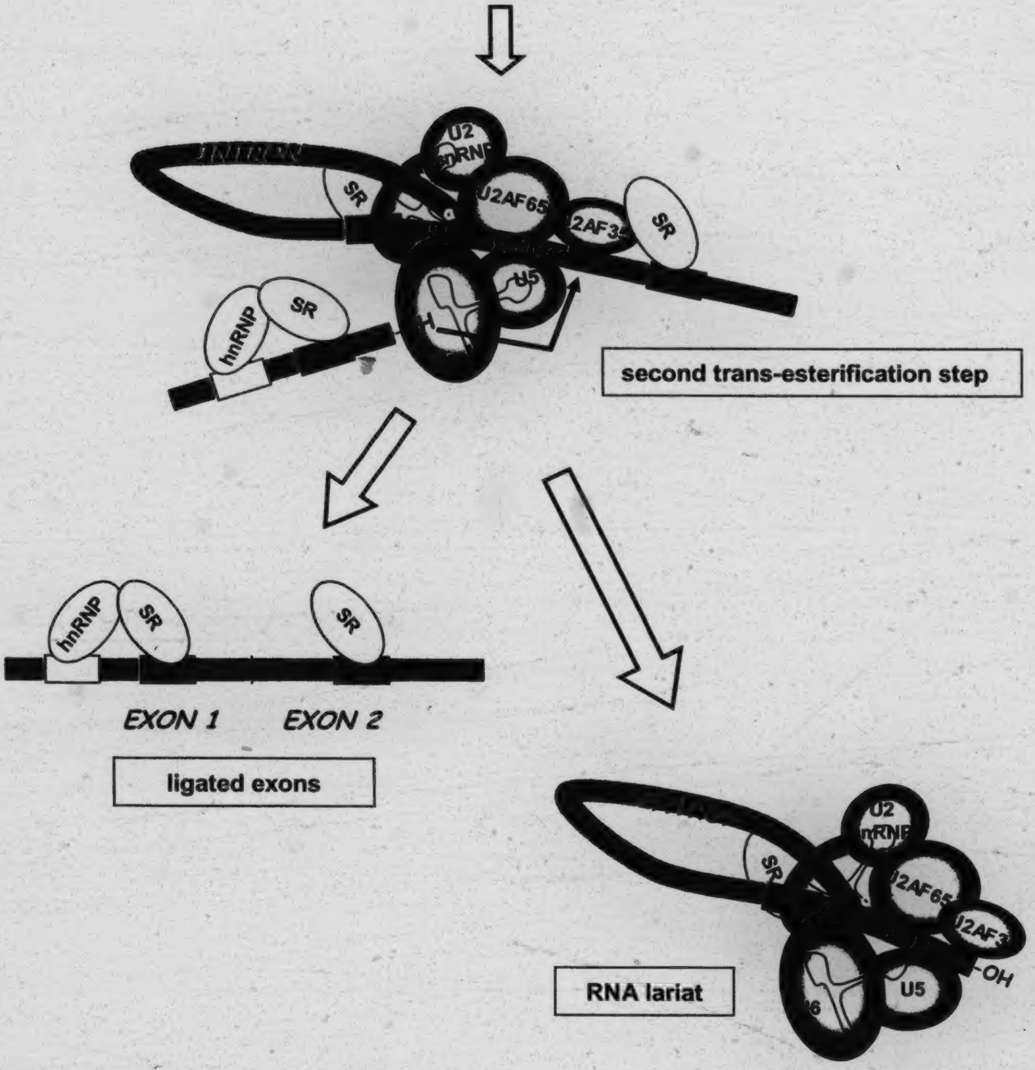

INTRON
U2 snRNP
SR
U2AF65
U2AF35
SR
U5
SR
hnRNP
second trans-esterification step
hnRNP
SR
SR
EXON 1
EXON 2
ligated exons
U2 snRNP
SR
U2AF65
U2AF35
U5
-OH
RNA lariat

Fig. 1.6B - Molecular mechanism of spliceosome assembly and RNA splicing *cont'd*

and the RNA is now ready for catalysis. The first trans-esterification step occurs when the 2'-OH group of the adenosine residue in the branchpoint sequence attacks the guanosine of the 5' splice site, thereby generating a free 3'-OH group at the 3'-end of the first exon and a branched lariat at the 5'-end of the intron. This is followed by the second trans-esterification step where the free 3'-OH group attacks the 5'-most residue of the second exon, thereby cleaving the RNA molecule at the 3' splice site. The resulting "spliced-out" intron is released as a lariat while the remaining two exons are ligated together (Patel and Steitz, 2003).

Although the U1 snRNP, U2 snRNP and U2AF associations have some sequence preference, their relative affinity to appropriate sites is reduced due to the degenerate nature of their recognition sequence. Recognition of exon-intron junctions and splice site usage for constitutive and alternative splicing require specific adjacently-located *cis* elements: splicing suppressors or enhancers. These are differentially bound by *trans* factors, categorized as heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine-arginine-rich (SR)/SR-related proteins. HnRNPs contain RNA-binding arginine-glycine-glycine (RGG) motifs which can bind exonic and intronic splicing suppressor elements (ESS and ISS) (Kalnina *et al.*, 2005). SR proteins contain one or two RNA-binding RRM motifs and the arginine-serine (R-S) sequence thought to be involved in protein-protein interaction and in promoting branchpoint sequence binding (Caceres and Kornblihtt, 2002; Huang and Steitz, 2005). SR proteins, for the most part, recognize exonic splicing enhancer elements (ESE), and to a lesser extent, intronic splicing enhancer elements (ISE) (Black, 2003; Kalnina *et al.*, 2005).

Additional SR-nonrelated proteins also bind ISEs (Pagani and Baralle, 2004). HnRNPs and SR proteins, when bound to their respective sequence elements, are thought to act through protein-protein interactions to modulate U1 snRNP, U2 snRNP and U2AF site recognition, splice site utilization and the stability of snRNP binding to pre-mRNA. While we have some insights as to how the interplay between snRNPs, hnRNPs and SR proteins regulates factor association at splice sites, the exact mechanism by which one particular splice site is recognized over another remains poorly understood.

1.2.2 Alternative splicing

Alternative splicing refers to the usage of alternative 5'-end and 3'-end splice sites within a pre-mRNA to form two or more different RNA products. Splice site usage is determined by three conditions (Smith and Valcarcel, 2000; Stamm *et al.*, 2005). First, the degeneracy of U1 snRNP (5' splice site), U2 snRNP (3' splice site) and U2AF (branchpoint) recognition sequences and the flexibility of these factors in binding to alternative or non-canonical sites. Second, the combination and concentrations of hnRNPs and SR-proteins bound to splicing suppressors and splicing enhancers, respectively. Third, phosphorylation/dephosphoryation of these regulatory proteins and ensuing alteration of protein-protein interaction profiles. It is estimated that 75% of all alternative splicing events result in a change in protein coding sequence through the addition, removal or modification of protein domains, motifs or post-translational targeting sequences (Okazaki *et al.*, 2002; Zavolan *et al.*, 2003).

These can have profound biochemical effects which may include: (i) changes in subcellular targeting motifs, (ii) altered or *de novo* binding properties affecting interaction with other proteins, nucleic acids or ligands, (iii) changes in enzymatic or signalling properties, and (iv) altered protein stability (Black, 2003; Stamm *et al.*, 2005). The remaining 25% of splice events account for changes in RNA activity by altering RNA-protein and RNA-RNA associations. The latter affect translation initiation/repression properties, subcellular RNA targeting, RNA stability and half-life, and differential targeting for nonsense-mediated decay (NMD) (Mignone *et al.*, 2002). Alternative splicing has been reported to occur in at least seven different patterns (illustrated in Fig. 1.7): (i) internal cassette-exon skipping/inclusion, (ii) mutual exclusion of alternative internal exons, (iii) alternative 5' splice site, (iv) alternative 3' splice site, (v) alternative 5'-end promoter, (vi) alternative 3'-end poly-adenylation site usage, and (vii) intron exclusion/retention (Black, 2003; Kalnina *et al.*, 2005; Matlin *et al.*, 2005). Alteration of splicing patterning is governed by cell type, developmental stage or by extracellular and intracellular environmental factors, such as receptor stimulation (hormones, growth factors, cytokines), cell stress (pH, hypoxia, heat shock) and changes in ion concentrations (Ca^{2+} levels) (Scotet and Houssaint, 1998; Stamm, 2002; Faustino and Cooper, 2003). As these conditions change, so do the patterns of alternative splicing.

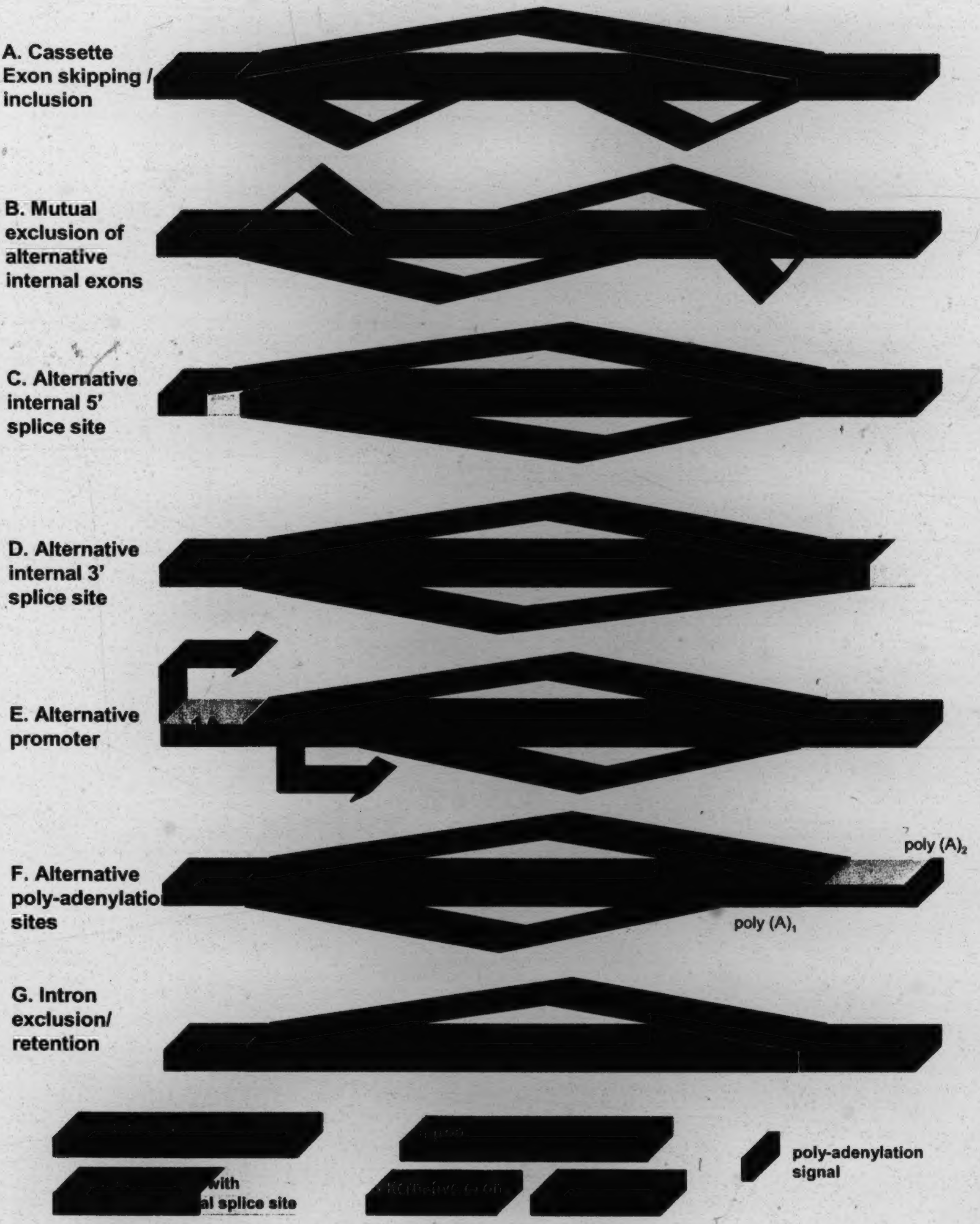

A. Cassette Exon skipping / inclusion
B. Mutual exclusion of alternative internal exons
C. Alternative internal 5' splice site
D. Alternative internal 3' splice site
E. Alternative promoter
F. Alternative poly-adenylation sites
poly (A)₂
poly (A)₁
G. Intron exclusion/ retention
with
al splice site
poly-adenylation signal

Fig. 1.7 - The seven patterns of alternative splicing

1.2.3 Alternative internal cassette exons

Inclusion/exclusion of internal cassette exons is one of the most commonly reported forms of alternative splicing (Modrek *et al.*, 2001). This pattern of splicing involves addition or subtraction of internal coding exons, thereby changing the protein coding sequence (Fig. 1.7A). This change can either be: (i) in-frame, thereby extending function through the addition or exclusion of sequence while maintaining some core protein functions, or (ii) shift codon frame, thereby altering sequences that are C-terminal to the internal cassette exon including the possible incorporation of a stop codon. An example of cassette exon inclusion/exclusion is illustrated in the alternative splicing of *Numb* in neuronal precursor cells. The p72 Numb isoform includes a 33 nt exon encoding an 11 aa motif which anchors Numb to the plasma membrane (PM) (Dho *et al.*, 1999). Alternative splicing and exclusion of this region creates a p71 Numb isoform which localizes to the cytoplasm (Verdi *et al.*, 1996; Zhong *et al.*, 1996; Salcini *et al.*, 1997).

A related pattern is the mutual exclusion of alternative exons, a pattern resulting in only one out of two or more adjacent exons being included in mature transcripts (Fig. 1.7B). This splicing pattern requires an additional level of complexity as factors that specify exclusion of one exon must be coordinated with those that specify inclusion of the adjacent exon. As a result of the intricate nature of this splicing pattern, few well-understood examples have been documented in the literature. Such a pattern type has been described in the alternative splicing of rat fibroblast growth factor receptor 2 (*FGFR2*). Factor recognition of a novel intronic splicing element (ISE/ISS-3) upstream of *FGFR2* exon IIIC is mediated through a non-canonical guanosine branchpoint residue (instead of adenosine) (Hovhannisyan and Carstens, 2005). This forces sequestering of exon IIIC into the RNA lariat and inclusion of exon IIIB to form *FGFR2-IIIB*. When the *FGFR2* transcript includes exon IIIC (*FGFR2-IIIC*), the guanosine branchpoint is not recognized and exon IIIB is excluded. Both exons IIIB and IIIC encode the C-terminal-half of an extracellular immunoglobulin-like domain, resulting in different ligand-binding preferences (Ornitz *et al.*, 1996). FGFR2-IIIB is expressed in epithelial cells whereas FGFR2-IIIC is expressed in mesenchymal cells (De Moerlooze *et al.*, 2000). Tissue-specific expression of these isoforms is crucial for intracellular communication and proper organ development.

1.2.4 Alternative 5'- and 3'-end splice sites

Usage of alternative internal 5' or 3' splice sites represents another way of varying particular sequences or domains inherent to a specific exon and/or alter downstream sequences (Figs. 1.7C, 1.7D). In this splice pattern, instead of recognizing the presumptive splice site at intron-exon (3' splice site) or exon-intron (5' splice site) junctions, weak or cryptic splice sites within the exon are recognized. This is mediated, in part, by the recognition of alternative branchpoint sequences and splicing enhancer/suppressor elements. Alternative internal 5' and 3' splice site selection creates isoforms with exonic N-terminal and C-terminal truncations, respectively. If selection of this splice site changes the frame of the truncated exon with relation to the rest of the isoform coding region, it may either create or destroy specific biochemical domains inherent to that exon and alter the remaining coding frame (and protein sequence) C-terminal to the splice site. This alteration may include protein truncation by introduction of a premature stop codon.

Examples illustrating alternative 5'-end splice site selection include the pro- and anti-apoptotic forms of Bcl-x (Minn *et al.*, 1996). In A549 lung adenocarcinoma cells, predominant expression of the anti-apoptotic Bcl-x(L) (large) isoform helps to deregulate cellular apoptosis, thereby allowing cell survival (Boise *et al.*, 1993). Expression of Bcl-x(L) is mediated by inclusion of *Bcl-x* exon 2. Addition of cell-permeable D-e-C_6 ceramide shifts Bcl-x action from anti-apoptotic to pro-apoptotic by enhancing expression of the dominant negative Bcl-x(s) (small) isoform (Minn *et al.*, 1996; Chalfant *et al.*, 2002). Ceramide acts to dephosphorylate SR proteins (SRp70, SRp55, SRp40, and SRp30) through protein phosphatase 1 (PP1) induction, thereby altering their affinity for two splicing elements and recognition of an internal 5' splice site within *Bcl-x* exon 2. (Chalfant *et al.*, 1999; Chalfant *et al.*, 2001; Chalfant *et al.*, 2002; Massiello *et al.*, 2004). As a consequence, the N-terminal half of exon 2 is ligated to exon 3 to form *Bcl-x(s)* and the C-terminal half of exon 2 is excluded from the mature transcript. There may be clinical applications to these observations as treatment of prostate and breast cancer cells with an antisense oligonucleotide against the *Bcl-x(L)*-specific upstream 5' splice site prevents recognition of this site in splicing (Mercatante *et al.*, 2001). This forces recognition of the internal (downstream) 5' splice site, thereby forming the pro-apoptotic *Bcl-x(s)* splice form.

A paradigm of alternative splicing has emerged through the study of exon 9 alternative splicing in cystic fibrosis transmembrane conductance regulator (*CFTR*), the gene responsible for cystic fibrosis. One population of *CFTR* mutations results in aberrant skipping of exon 9, thereby producing a non-functional protein (Chu *et al.*, 1993; Chillon *et al.*, 1995; Mak *et al.*, 1997; Cuppens *et al.*, 1998; Larriba *et al.*, 1998). A recent study has demonstrated that the alternative splicing factor, TIA-1, promotes exon 9 inclusion (Zuccato *et al.*, 2004). Interestingly, mutation of the constitutive 3' splice site at the intron-exon 9 junction results in TIA-1-mediated recognition of a previously unknown cryptic 3' splice site in *CFTR* exon 9. The resulting isoform excludes the N-terminal portion of exon 9 and retains only the C-terminal end, thereby encoding a non-functional CFTR protein. Aberrant splicing of exon 13 has also been observed in patients harboring exonic D648V, E664X and T665S mutations (Aznarez *et al.*, 2003). These mutations act to improve polypyrimidine tract recognition of two independent cryptic 3' splice sites in CFTR exon 13. Recognition of these splice sites results in exclusion of a N-terminal portion of exon 13 and inactivation of the CFTR gene product in cystic fibrosis patients.

1.2.5 Alternative 5'-end promoter

Usage of alternative 5' leader exons from within alternative downstream promoters is related to the same splicing pattern that recognizes alternative internal 5' splice sites (Fig. 1.7E). In this case, an upstream exon 1A is transcribed from its appropriate promoter to produce a pre-mRNA form. A second transcript is transcribed from a downstream promoter, producing a shorter version of the pre-mRNA which excludes upstream exon 1A and includes an alternate downstream exon 1B. Processing of the longer pre-mRNA results in exclusion of exon 1B and ligation of exon 1A with exon 2. Processing of the shorter pre-mRNA entails ligation of exon 1B with exon 2. If exons 1A, 1B and/or 2 have alternative AUG translation initiation codons, this type of splicing may result in different protein start sites, thus altering N-terminal sequence and function. Alternatively, the consequence of differential exon 1 usage may be the inclusion of different 5' UTR sequence elements and modulation of RNA activity.

Alternative promoter usage has been reported in protein 4.1R expression during erythroblast differentiation (Parra *et al.*, 2003). Three alternative exons 1 exist: 1A, 1B and 1C, all under independent promoters. Transcription at the start of either exon 1B or 1C results in their splicing with an alternative exon 2 splice site (2') located upstream of the constituitive exon 2 splice site. Ligation of exon 1B or 1C with the 2' splice site results in a transcript which utilizes an alternative AUG start codon thereby encoding a protein of 135 kDa. Although they have identical protein sequences, exon 1B- and exon 1C-containing transcripts have different 5' UTRs. Both transcripts are expressed at equally low levels in erythroid cells; however, exon 1C-containing transcripts are found in higher abundance in non-erythroid lineages compared to exon-1B-containing transcripts. In addition, exon 1A in exon 1A-containing transcripts has been shown to ligate to the downstream exon 2 splice site, thereby excluding the exon 2' region and its alternative AUG start codon. Instead, an AUG start site located in exon 4 is used to encode an exon-1A-containing 80 kDa protein. Exon-1A-containing RNA and protein are found at low levels in erythroblasts and increase with erythroid differentiation (Koury *et al.*, 1986; Hanspal *et al.*, 1992; Parra *et al.*, 2003).

1.2.6 Alternative 3'-end poly-adenylation

Recognition of alternative 3'-end poly-adenylation sites is thought to alter RNA activity primarily through modulation of 3'-UTR regulatory elements (Fig. 1.7F). This splicing pattern is similar to that of alternative recognition of internal 3' splice site with the exception that splicing occurs at the end of the transcript, normally specifying the final exon and terminal 3'UTR. In some cases, the C-terminal protein sequence may also be altered if splicing occurs within the protein coding region. Recognition of the final 3' splice site in a pre-mRNA usually involves a termination signal by which cleavage and poly-adenylation factors recognize, bind, cleave and poly-adenylate the transcript as one of the final steps in RNA maturation. This final RNA processing step helps to mediate subcellular RNA localization and regulate RNA degradation. Depending on the location and combination of 3'-end regulatory elements, differing rates of RNA degradation are possible through interactions between regulatory RNA-binding proteins and cleavage, poly-adenylation and RNA degradation machineries. Alternative poly-adenylation can therefore modulate RNA stability and RNA activity.

An interesting example comes from the expression of a novel isoform of glial fibrillary acidic protein, GFAPε (Nielsen *et al.*, 2002). The GFAPε splice form is produced as a result of recognition of an alternative branchpoint sequence and 3' splice acceptor sequence within *GFAP* intron 7. This allows for the in-frame splicing of alternative exon 7A, an exon which contains an alternative poly-adenylation signal. In GFAPα, the constituitive form of GFAP, exon 7 is directly spliced to exons 8 followed by splicing to exon 9, with a poly-adenylation signal contained within exon 9. Thus, the GFAPε C-terminus encodes a tail region that differs from that of GFAPα. While this alternative C-terminus does not interfere with GFAPε integration and activity within GFAP-containing intermediate filaments, it facilitates a novel interaction with presinilin-1, a membrane bound protease. Presenilin-1 and GFAPα, however, do not interact. Another major difference between GFAPε and GFAPα is that *GFAPε* mRNAs are found at 20-fold reduced levels compared to *GFAPα* mRNAs. While this difference in transcript levels could be attributed to reduced rates of alternative splicing of the *GFAPε* form, it is also likely that alternative poly-adenylation alters *GFAPε* transcript stability as compared to *GFAPα* mRNA.

1.2.7 Introns as exons

The last splicing pattern type is intron retention (Fig. 1.7G). This pattern is the least studied, and more often than not, the most overlooked as these splice variants are often discounted as unspliced pre-mRNA or splicing intermediates. Intron retention can have similar consequences to those described for the other splicing patterns, with the most common being alteration of protein coding sequences C-terminal to the splicing event. A well-known example of intron retention is found in the expression of the RET tyrosine kinase (Le Hir *et al.*, 2002). A splice form of RET that retains intron 2 is enriched in some familial and sporadic cases of pheochromocytomas. This form encodes a protein with an in-frame stop codon, resulting in a constitutively active form of RET due to protein truncation.

1.3 THE REELIN-DISABLED 1 SIGNALING PATHWAY

1.3.1 Reelin-Disabled 1 signaling in cortical development: the reeler phenotype

Development of the cerebral cortex and cerebellum requires the correct positioning of cortical plate neurons. A major pathway that governs cortical plate neuronal cell migration is the Reelin-Disabled 1 pathway. This pathway is comprised of four key components: Reelin, an extracellular matrix protein (Curran and D'Arcangelo, 1998); the Reelin receptors, apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) (Trommsdorff *et al.*, 1999); and Disabled-1 (Dab1), a cytosolic adaptor protein associated with Reelin-receptor cytoplasmic tails (Rice *et al.*, 1998).

Mice harboring mutions in *Reelin* (*reeler*), *Dab1* (*scrambler/yotari/mdab1-1*) or both *ApoER2* and *VLDLR* (*ApoER2*$^{-/-}$*VLDLR*$^{-/-}$) have similar cortical lamination defects in the brain, characterized by inversion of neuronal layers, abnormal neuronal positioning, defects in cell body orientation and aberrant positioning of their nerve fibers (Stanfield and Cowan, 1979; D'Arcangelo *et al.*, 1995; Gonzalez *et al.*, 1997; Sheldon *et al.*, 1997; Ware *et al.*, 1997; Yoneshima *et al.*, 1997; Howell *et al.*, 1997b; D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999; Drakew *et al.*, 2002; Niu *et al.*, 2004). Cerebellar defects include hypoplasia (Figs. 1.8A-I), malpositioned Purkinje cells and reduced Purkinje cell number (Goffinet *et al.*, 1984; Hadj-Sahraoui *et al.*, 1996; Magdaleno *et al.*, 2002). *Reelin*$^{-/-}$ and *Dab1*$^{-/-}$ mutant mice also show malformation of the radial glial cell scaffold (Forster *et al.*, 2002; Hartfuss *et al.*, 2003). Radial glial cells are involved in anchoring, positioning and migration of neurons during cortical development. As a result of these neuronal positioning defects, *reeler* mice display reeling gait and severe ataxia (Figs. 1.8J, 1.8K) (Goffinet, 1979; Goffinet, 1984; Magdaleno *et al.*, 2002). In humans, *Reelin* mutations are associated with autosomal recessive lissencephaly (meaning “smooth brain”), a neuronal migration disorder characterized by cerebellar hypoplasia, ataxia, severe cognitive delay, hypotonia and seizures (Hong *et al.*, 2000; Guerrini and Filippi, 2005). *Reelin* mutations are also associated with schizophrenia, autism, bipolar disorder and major depression (Fatemi, 2005). Recently, a novel human mutation in the *VLDLR* gene was identified (Boycott *et al.*, 2005). Individuals with this mutation present with some of the same symptoms observed in individuals with Reelin mutations; ie. autosomal cerebellar hypoplasia resulting in cerebellar ataxia and mental retardation. *VLDLR* represents only the second component of the Reelin-Dab1 pathway with documented mutation in humans. To date, *Dab1* mutations have not been reported in humans (Meyer *et al.*, 2003).

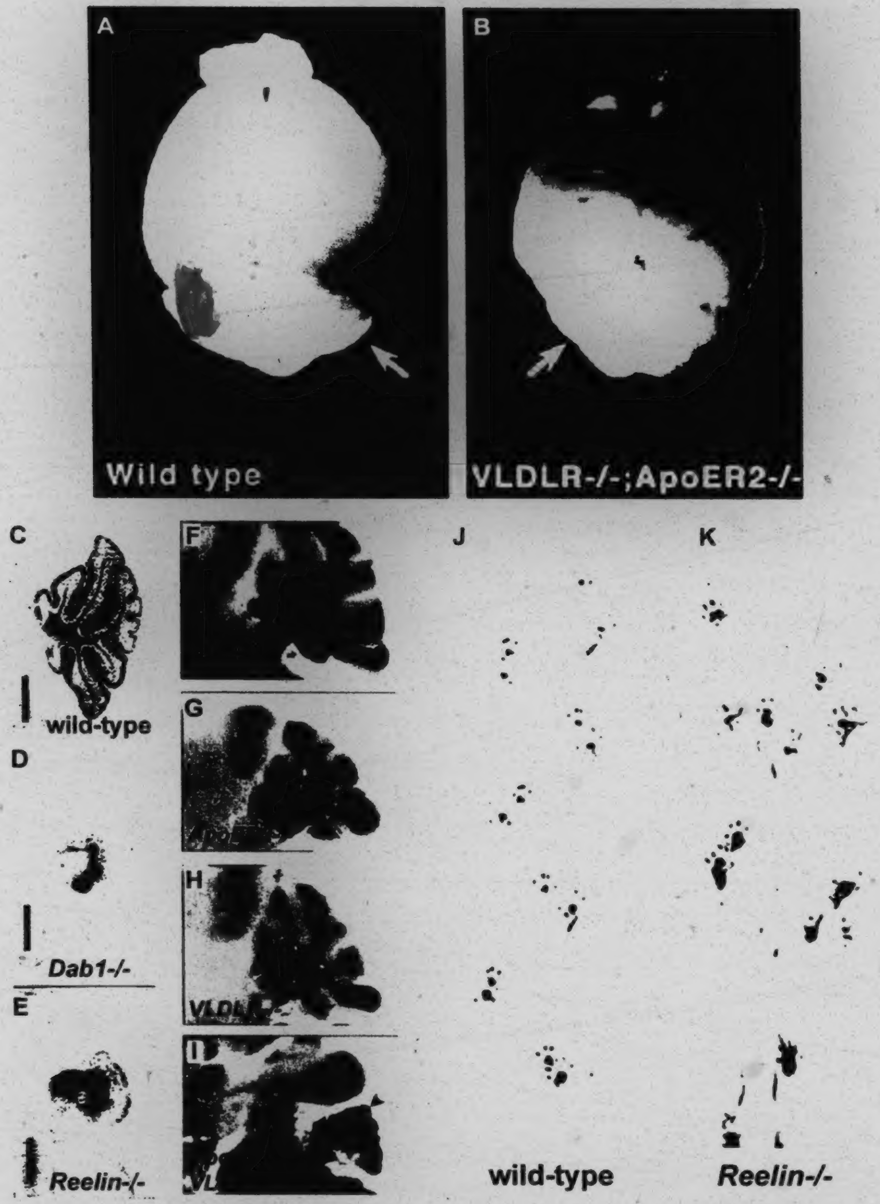

A
B
Wild type
VLDLR-/-;ApoER2-/-
C
F
J
K
wild-type
G
D
Dab1-/-
H
E
I
Reelin-/-
wild-type
Reelin-/-

Fig 1.8 - Cerebellar **(A-I)** and ataxic phenotypes **(J,K)** of mice deficient in Reelin-Dab1 signaling. The arrow in **(B)** indicates the hypoplastic nature of cerebella derived from mutant mice as compared to wild type mice **(A)**. **(J,K)** Mouse footprint tracings of wild-type **(J)** and *reeler* mice **(K)**

Fig. 1.8A,B, F-I modified from Trommsdorff et. al., 1999; Fig. 1.8C-E modified from Yoneshima et. al., 1997; Fig. 1.8J,K modified from Magdaleno et. al., 2002

1.3.2 Overview of Reelin-mediated activation of Dab1

Reelin is an extracellular matrix glycoprotein with serine protease activity (D'Arcangelo *et al.*, 1997; Quattrocchi *et al.*, 2002). Full-length Reelin is composed of 3461 amino acids (aa) and is comprised of eight Reelin-repeat domains, each of approximately 350 aa, which contain an EGF-like motif flanked by two sub-repeats (Fig. 1.9) (de Bergeyck *et al.*, 1998). Activation of secreted Reelin requires two proteolytic cleavage events between Reelin-repeats 2-3 and 6-7 (arrows in Fig. 1.9) (Lambert de *et al.*, 1999; Jossin *et al.*, 2004). At the N-terminus lies a unique region (denoted as H in Fig. 1.9) which contains the CR-50 epitope. This epitope is required for Reelin multimerization (D'Arcangelo *et al.*, 1997; Kubo *et al.*, 2002). Mutation of this region or blocking with the anti-Reelin CR-50 antibody disrupts Reelin-Reelin electrostatic interactions, prevents its binding to receptors and prevents Reelin-mediated cortical development (Miyata *et al.*, 1997; Nakajima *et al.*, 1997; Utsunomiya-Tate *et al.*, 2000). Furthermore, the central Reelin fragment (between repeats 3-6) is also essential for receptor binding and Reelin-mediated signaling (Jossin *et al.*, 2004); however, its involvement with the N-terminal Reelin fragment in mediating Reelin-receptor binding and signaling remains poorly understood.

In the cerebral cortex, Reelin is secreted by Cajal-Retzius (CR) neurons in the cortical marginal zone (layer I). Cajal-Retzius cells are the first neuronal cell type to differentiate in the developing brain (Marin-Padilla and Marin-Padilla, 1982; Derer and Derer, 1990; D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995; D'Arcangelo *et al.*, 1997). Reelin is also expressed in cortical GABAergic

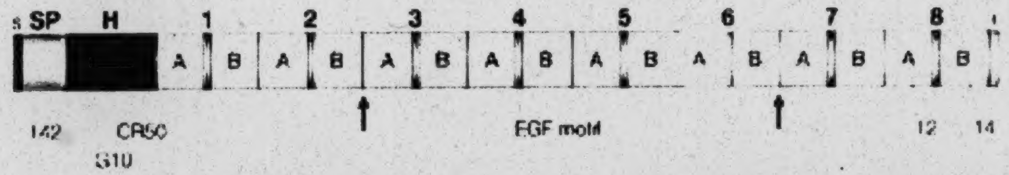


Fig. 1.9 - Schematic of the Reelin protein. Reelin is composed of 8 Reelin-repeat domains, each comprised of an EGF-like motif and flanked by A and B sub-repeats. At the N-terminus lies a unique motif (H) containing the CR-50 epitope, a site for Reelin-multimerization which can be blocked through binding of the CR-50 antibody. Two other epitopes, 142 and G10, are indicated and correspond to the antigenic regions of two additional Reelin antibodies. Arrows denote cleavage sites required for Reelin proteolysis and activation. S denotes a signal peptide, SP denotes an F-spondin-like domain.

Fig. 1.9 modified from Tissir and Goffinet, 2003.

interneurons (layers II-IV) and cerebellar granule layer cells (D'Arcangelo *et al.*, 1995; Impagnatiello *et al.*, 1998). Neighboring cells such as the presumptive pyramidal neurons and migrating cortical plate neurons, located in the ventricular zone, are targets for the Reelin architectonic signal as these cell types express the Reelin receptors and the cytoplasmic adaptor protein, Dab1 (Tissir and Goffinet, 2003).

A variety of surface receptors bind extracellular Reelin including ApoER2, VLDLR, alpha3beta1 integrin and cadherin-related neuronal receptor (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Senzaki *et al.*, 1999; Dulabon *et al.*, 2000). In the Reelin-Dab1 pathway, Reelin signals that promote neuronal migration are transduced to Dab1 via ApoER2 and VLDLR (Hiesberger *et al.*, 1999). ApoER2 and VLDLR each have three functional domains: the extracellular Reelin-binding domain, a transmembrane bound domain, and a tail domain containing the F(phe)-x(any)-N(asn)-P(pro)-x(any)-Y(tyr) docking site. Dab1, a cytosolic adaptor protein, associates with conserved cytoplasmic FxNPxY docking sites within ApoER2 and VLDLR receptor tails in a Reelin-dependant manner (Trommsdorff *et al.*, 1998; Howell *et al.*, 1999b; Morimura *et al.*, 2005). Binding of the Reelin multimer to VLDLR and ApoER2 induces receptor clustering, Dab1 recruitment to the plasma membrane, Dab1 oligomerization and basal phosphorylation of Dab1 at tyrosine-198 (Hiesberger *et al.*, 1999; Keshvara *et al.*, 2001; Magdaleno *et al.*, 2002; Benhayon *et al.*, 2003; Strasser *et al.*, 2004; Morimura *et al.*, 2005). Oligomerization is critical to the activation of Dab1 as treatment with ApoER2/VLDLR-specific bivalent agents which promote Reelin-independent receptor clustering, induces Dab1-Dab1 associations and basal Dab1 tyrosine phosphorylation (Strasser *et al.*, 2004). The exact biochemical mechanism of basal Dab1 activation is not known - it may occur through Dab1 autophosphorylation or involve receptor tyrosine kinases (RTKs) or nonreceptor tyrosine kinases (NTKs). However, recent reports implicate Dab1-phosphoinositide binding as being crucial to basal Dab1 tyrosine phosphorylation (Huang *et al.*, 2005; Stolt *et al.*, 2005; Xu *et al.*, 2005).

Dab1 transduces the Reelin signal by activating members of the Src-family tyrosine kinase (SFK) family such as Fyn and Src, as well as PI3K/Akt serine/threonine kinases (Bock and Herz, 2003; Arnaud *et al.*, 2003b). Activated SFKs potentiate and hyperphosphorylate Dab1 which, in turn, initiates a self-regulating SFK tyrosine kinase signaling cascade which is thought to phosphorylate downstream effectors involved in cytoskeletal organization and neuronal migration (Bock and Herz, 2003; Arnaud *et al.*, 2003b). Functional perturbation of components of the Reelin-Dab1 pathway using Reelin-inhibiting antibodies (such as CR-50), receptor antagonists (RAP) or inhibitors of Dab1 phosphorylation (SFK-inhibiting protein phosphatase-2) prevents neuronal dendrite outgrowth (Ogawa *et al.*, 1995; D'Arcangelo *et al.*, 1997; Hiesberger *et al.*, 1999; Bock and Herz, 2003; Arnaud *et al.*, 2003b; Niu *et al.*, 2004). Activation of Abelson family tyrosine kinases (Abl and Arg) is also implicated in Dab1 function. While some reports suggest a direct interaction between Dab1 and Abl (Howell *et al.*, 1997a), others suggest that Abl family kinases indirectly associate with Dab1 through SFKs or SFK-mediated recruitment of adaptor proteins (Feller, 2001). Combined, Reelin-mediated Dab1 phosphorylation is shown to regulate a variety of pathways (Figs 1.10, 1.11) including PI3K/Akt/GSK-3β, N-WASP, Nckβ, CrkII/Dock/Rac and CrkL/C3G/RAP1 (Howell *et al.*, 1999b; Beffert *et al.*, 2002; Ballif *et al.*, 2003; Benhayon *et al.*, 2003; Bock and Herz, 2003; Bock *et al.*, 2003; Pramatarova *et al.*, 2003; Arnaud *et al.*, 2003b; Ballif *et al.*, 2004; Chen *et al.*, 2004; Suetsugu *et al.*, 2004). These effectors are thought to function synergistically in additive or opposing ways to modulate intracellular cytoskeletal dynamics and cellular morphology resulting in Reelin-mediated neuronal migration and neuritogenesis.

1.3.3 Identification of Disabled-1

Disabled was originally identified in *Drosophila* where it was found to bind Abl. In *Drosophila*, Disabled is involved in Abl-mediated eye (ommatidial) development and axon formation (Gertler *et al.*, 1989; Gertler *et al.*, 1993; Le and Simon, 1998). The mammalian counterpart of Disabled, called Dab1, was first identified through yeast two-hybrid analysis, where it was found to interact with the Src tyrosine kinase (Howell *et al.*, 1997a). Dab1 cDNAs have now been identified or isolated from a variety of higher eukaryotes, including nematode, lizard, turtle, mouse, rat, chicken, monkey and human. It should be noted that Reelin has been identified in all vertebrates tested to date, suggesting an important evolutionary-conserved role for the Reelin-Dab1 signaling pathway in neuronal development of higher-ordered organisms (Bar *et al.*, 2003; Tissir and Goffinet, 2003).

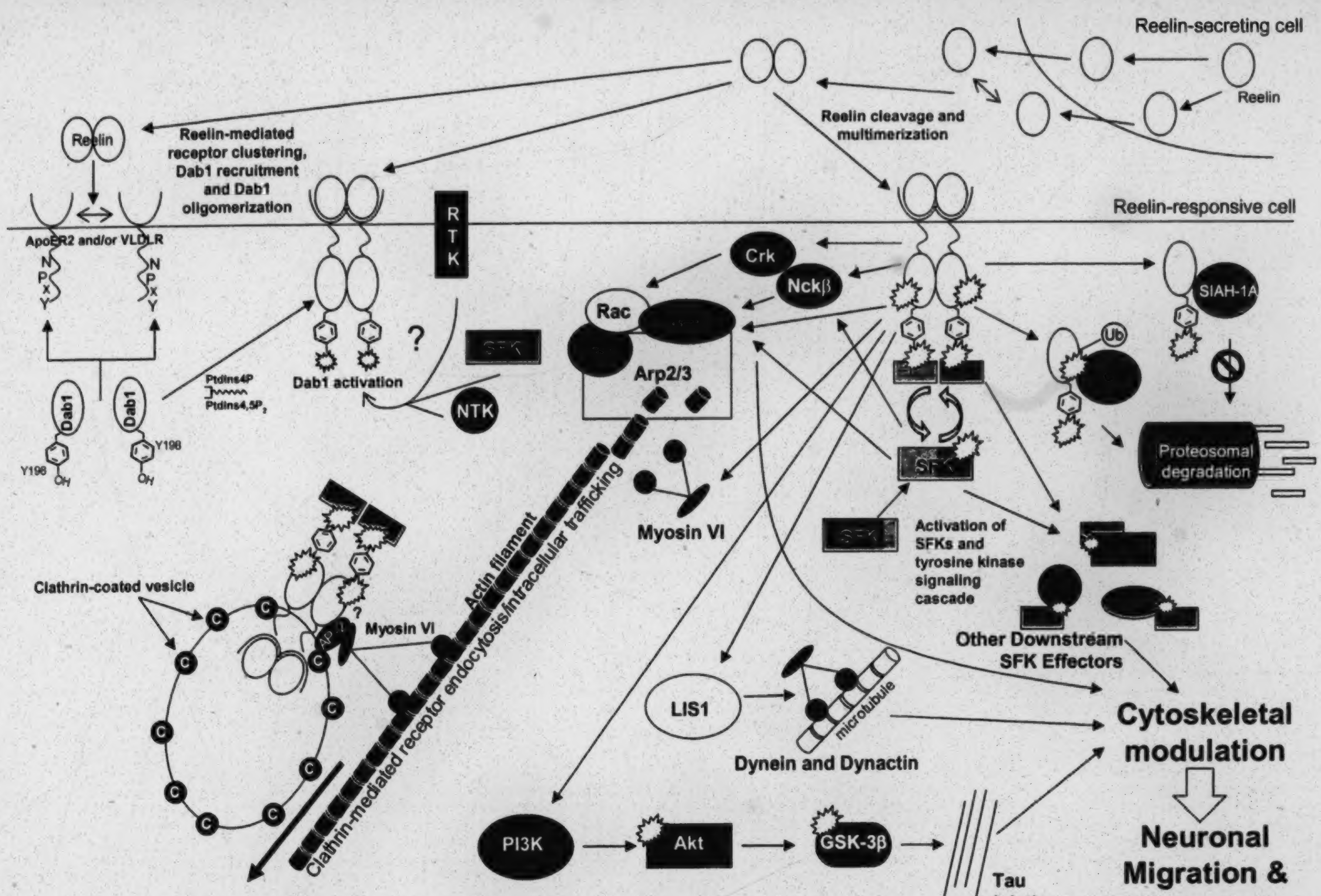

Reelin-secreting cell
Reelin
Reelin cleavage and multimerization
Reelin
Reelin-mediated receptor clustering, Dab1 recruitment and Dab1 oligomerization
Reelin-responsive cell
ApoER2 and/or VLDLR
N
P
x
Y
N
P
x
Y
Dab1
Dab1
Y198
Y198
OH
OH
PtdIns4P
PtdIns4,5P$_2$
R T K
Dab1 activation
?
SFK
NTK
Crk
Nckβ
Rac
Arp2/3
Myosin VI
SIAH-1A
Ub
Proteosomal degradation
SFK
SFK
Activation of SFKs and tyrosine kinase signaling cascade
Other Downstream SFK Effectors
Actin filament
Clathrin-mediated receptor endocytosis/intracellular trafficking
Clathrin-coated vesicle
C
AP
?
Myosin VI
LIS1
microtubule
Dynein and Dynactin
Cytoskeletal modulation
Neuronal Migration & Differentiation
PI3K
Akt
GSK-3β
Tau modulation

Figure 1.10 - Overview of Reelin-Dab1 signaling

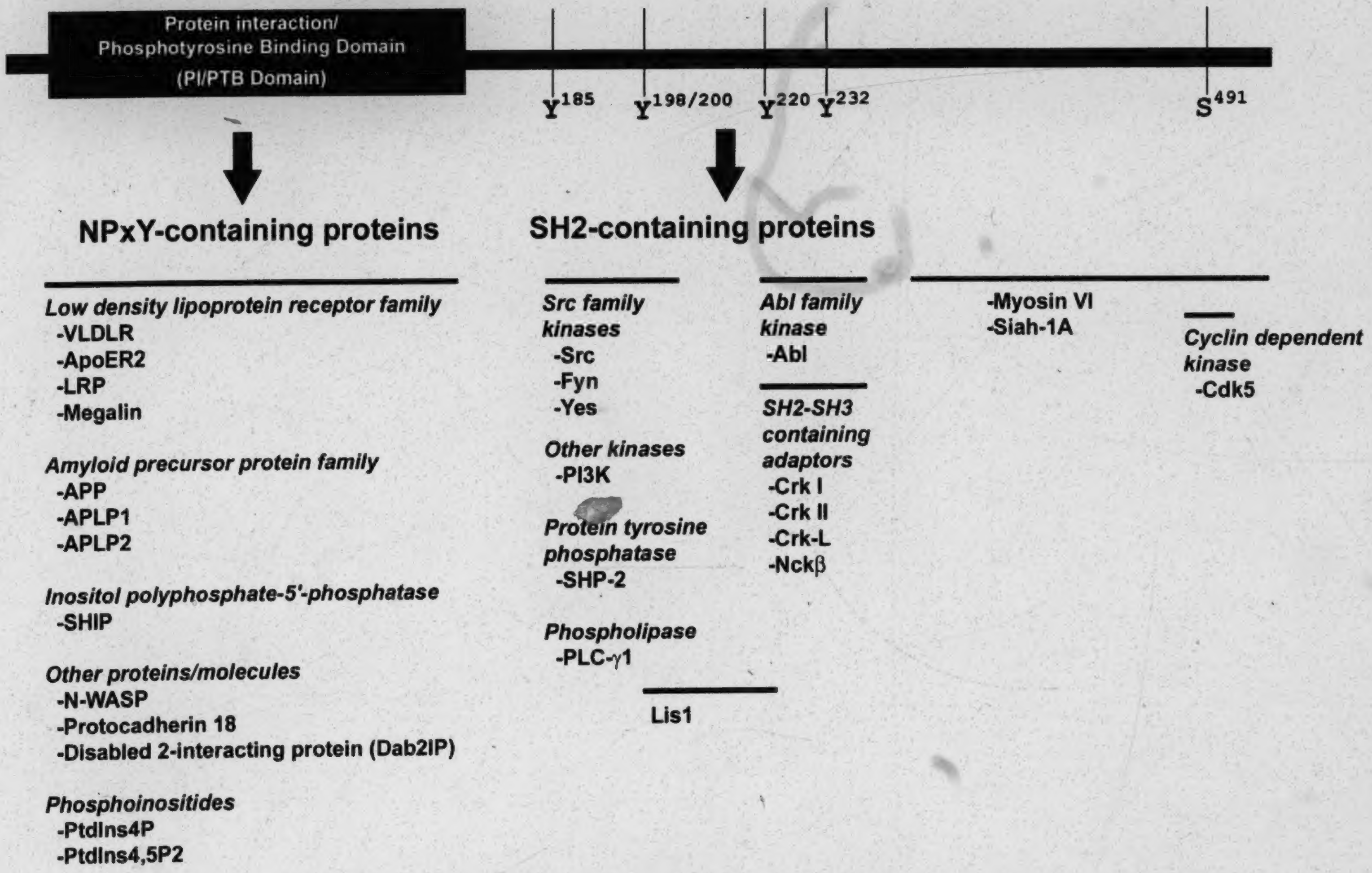

Protein interaction/
Phosphotyrosine Binding Domain
(PI/PTB Domain)
Y185
Y198/200
Y220
Y232
S491
NPxY-containing proteins
SH2-containing proteins
Low density lipoprotein receptor family
-VLDLR
-ApoER2
-LRP
-Megalin
Amyloid precursor protein family
-APP
-APLP1
-APLP2
Inositol polyphosphate-5'-phosphatase
-SHIP
Other proteins/molecules
-N-WASP
-Protocadherin 18
-Disabled 2-interacting protein (Dab2IP)
Phosphoinositides
-PtdIns4P
-PtdIns4,5P2
Src family kinases
-Src
-Fyn
-Yes
Other kinases
-PI3K
Protein tyrosine phosphatase
-SHP-2
Phospholipase
-PLC-γ1
Lis1
Abl family kinase
-Abl
SH2-SH3 containing adaptors
-Crk I
-Crk II
-Crk-L
-Nckβ
-Myosin VI
-Siah-1A
Cyclin dependent kinase
-Cdk5

Fig. 1.11 - Dab1 structure and molecular interactions

To date, two mammalian *Disabled* orthologues have been identified, *Dab1* and *Dab2* (Xu *et al.*, 1995; Howell *et al.*, 1997a). Dab1 and Dab2 share 63% overall similarity, with 82% similar residues within their conserved N-terminal PI/PTB domains. Both function as cytoplasmic adaptors. Dab1 is primarily expressed in cells of neuroectodermal origin and undergoes tyrosine phosphorylation in response to signal stimuli (Hiesberger *et al.*, 1999; Benhayon *et al.*, 2003). In contrast, Dab2 is ubiquitously expressed and is thought to undergo serine/threonine phosphorylation (Xu *et al.*, 1995). Based on *Dab2* mouse knock-outs and tissue expression patterns, Dab2 is required for visceral endoderm development and for the proper functioning of the mature renal proximal tubule (Morris *et al.*, 2002b). Dab2 is also a candidate tumour suppressor through uncoupling of c-Fos function, inhibition of MAP kinase signaling and induction of integrin-dependent *anoikis* (apoptosis of cells that have lost contact with the extracellular matrix) (He *et al.*, 2001; Smith *et al.*, 2001; Wang *et al.*, 2001a). Dab2, as a tumour suppressor, has been implicated in a variety of epithelial tumours such as ovarian, breast, gastrointestinal, prostate and lung (Sheng *et al.*, 2000; Wang *et al.*, 2001a; Kleeff *et al.*, 2002; Wang *et al.*, 2002b; Yano *et al.*, 2005).

1.3.4 Dab1 gene structure, protein domains and isoforms

The structure of human and mouse *Dab1* genes are similar but complex in nature. The mammalian *Dab1* gene spans 1100 kb and contains 14 main coding exons, as well as multiple 5' exons and some alternative internal coding exons

(DeSilva *et al.*, 1997; Royaux *et al.*, 1997; Bar *et al.*, 2003). The major form of *Dab1* mRNA encodes a protein of 555 aa (Dab1^{555}) (Howell *et al.*, 1997a; Bar *et al.*, 2003). Dab1^{555} (henceforth referred to as Dab1) is comprised of three major protein domains (Fig. 1.11): (i) a conserved N-terminal 'protein interaction/phosphotyrosine-binding domain' (PI/PTB) (170 aa) which binds to cytoplasmic F/YxNPxY docking sites found on Reelin-receptors and other select proteins (Trommsdorff *et al.*, 1998; Howell *et al.*, 1999b), (ii) a centralized tyrosine-rich domain containing five tyrosine (Y) residues located within four conserved tyrosine phosphorylation motifs: Y^{185}QTI and Y^{198}QYI which correspond to consensus Src-family tyrosine kinase recognition sites and Y^{220}QVP and Y^{232}DVP which correspond to consensus Abl family tyrosine kinase recognition sites (Songyang *et al.*, 1993; Howell *et al.*, 1997a; Howell *et al.*, 2000), and (iii) a C-terminal domain (310 aa) involved in Dab1-protein interactions and implicated in the modulation of Reelin-Dab1-dependent signaling and regulation of Dab1 activity (Herrick and Cooper, 2002; Keshvara *et al.*, 2002; Morris *et al.*, 2002a).

In addition to Dab1^{555}, two alternatively spliced isoforms, Dab1^{271} and Dab1^{217}, have been documented in the literature (Howell *et al.*, 1997a). Both of these isoforms contain the N-terminal PI/PTB domain; however, these splice variants have alternative 3' exons which encode alternative C-termini (Howell *et al.*, 1997a). Dab1^{271} includes all tyrosine phosphorylation motifs followed by inclusion (after serine-241) of a short alternative exon (encoding 30 aa) with an in-frame stop codon. Dab1^{217} includes only the first two tyrosine phosphorylation motifs, Y185 and Y198, followed by inclusion (after glutamine-199) of a short alternative exon (encoding 18 aa) with an in-frame stop codon. It is important to note that exon insertion in Dab1^{217} after glutamine-199 (Y^{198}QYI→Y^{198}QVI) does not alter the SFK tyrosine phosphorylation recognition motif (YQxI). There is some evidence suggesting that expression of these C-terminally truncated Dab1 isoforms may be associated with cell-specific differences in the extent of neuronal migration during cortical development (Herrick and Cooper, 2002).

1.3.5 The Dab1-PI/PTB domain: association with F/YxNPxY docking sites and phosphoinositides

The Dab1 PI/PTB domain binds two major classes of transmembrane protein families: the FxNPVY-containing low density lipoprotein receptor family (ApoER2, VLDLR, LRP) and the YENPVY-containing amyloid precursor protein family (APP, APLP1 and APLP2) (Trommsdorff *et al.*, 1998; Homayouni *et al.*, 1999; Howell *et al.*, 1999b). Dab1-PI/PTB-bound cytoplasmic proteins include SHIP, N-WASP, Dab2-interacting protein (DAB2IP) and the LDLR-like protein megalin.

The Dab1-PI/PTB is structurally similar to that of the classical PTB-containing Shc (Bork and Margolis, 1995). This domain associates with a variety of membrane-bound receptors that contain F/YxNPxY docking sites located on receptor cytoplasmic tails (Trommsdorff *et al.*, 1998; Howell *et al.*, 1999b). In addition, this domain also binds to select cytoplasmic proteins that contain similar docking sites. Compared to other PI/PTB domains, the Dab1-PI/PTB domain is unique in that it preferentially binds unphosphorylated F/YxNPxY motifs, as opposed to Shc which binds tyrosine phosphorylated NPxY (van der *et al.*, 1996; Homayouni *et al.*, 1999). Furthermore, structural studies show that the Dab1-PI/PTB domain contains a group of N-terminal basic amino acids which make up a lipid-binding pleckstrin homology (PH) domain (Howell *et al.*, 1999b; Stolt *et al.*, 2003; Yun *et al.*, 2003; Huang *et al.*, 2005). A number of lipid-containing molecules have been shown to bind to the PH domain of Dab1 including the two phosphoinositides: PtdIns4P and PtdIns4,5P_2. PtdIns4,5P_2 has previously been shown to be a critical second messenger in actin cytoskeletal organization and synaptic vesicle trafficking (Martin, 2001). Mice deficient for PtdIns4,5P_2 in the brain show early postnatal lethality due to defects in synaptogenesis (Di Paolo *et al.*, 2004). Dab1-PI/PTB binding to F/YxNPxY docking sites and to phospholipids occurs independently of each other and is free of steric hindrance (Howell *et al.*, 1999b). Furthermore, Dab1 membrane localization and basal tyrosine phosphorylation are both dependent on Dab1-PI/PTB interactions with phospholipids and Reelin-receptors (Huang *et al.*, 2005; Stolt *et al.*, 2005; Xu *et al.*, 2005). Mutation of a critical basic residue (K45Q) within the Dab1-PI/PTB reduces phosphoinositide binding and membrane associations without inhibiting Dab1-VLDLR interactions (Xu *et al.*, 2005). This mutation also reduces both Dab1 basal tyrosine phosphorylation and Reelin-stimulated SFK-mediated Dab1 tyrosine phosphorylation. Conversely, mutation of serine-114 within the Dab1-PI/PTB (S114T) disrupts Dab1-VLDLR associations without affecting Dab1-phosphoinositide associations. This mutant shows reduced Reelin-dependent tyrosine phosphorylation while basal Dab1 tyrosine phosphorylation is unaffected.

Production of intracellular PtdIns4,5P_2 and PtdIns3,4,5P_3 occurs through an enzymatic equilibrium between phosphatidylinositol 3'-kinase (PI3K) and the phosphatase PTEN. PI3K phosphorylates PtdIns4,5P_2 at the 3' position to form PtdIns3,4,5P_3, while PTEN hydrolyzes PtdIns3,4,5P_3 to PtdIns4,5P_2 (Rameh and Cantley, 1999; Kalesnikoff *et al.*, 2003). Interestingly, PI3K is a downstream effector of activated Dab1 and activation of the PI3K pathway (PI3K/Akt/GSK-3β/Tau) is implicated in microtubule-mediated cytoskeletal reorganization and neuronal migration (Beffert *et al.*, 2002; Bock *et al.*, 2003). Dab1-PI/PTB also associates with the intracellular FxNPxY docking sites of SH2-containing 5'-inositol phosphatase (SHIP), an enzyme that decreases the intracellular pool of PtdIns3,4,5P_3 (by forming PtdIns3,4P_2) and negatively regulates the PI3K/Akt/GSK-3β pathway (Howell *et al.*, 1999b; Kalesnikoff *et al.*, 2003; Horn *et al.*, 2004). The interrelationship between SHIP, PI3K and Dab1 suggests an intriguing regulatory loop that may regulate Dab1 and PI3K activities through modulation of intracellular PtdIns4,5P_2 levels. PI3K enzymatic function may promote Dab1 activation through production of PtdIns4,5P_2. Conversely, SHIP may inactivate Dab1 by simultaneously inhibiting PI3K and hydrolyzing PtdIns3,4,5P_3 into PtdIns3,4P_2, thereby inhibiting the enzymatic kinase and eliminating the substrate involved in the synthesis of Dab1-activating PtdIns4,5P_2.

1.3.6 The Dab1-APP interaction

Although ApoER2 and VLDLR binding directly implicate Dab1 in the Reelin-Dab1 pathway, LRP and APP (including APP-like proteins APLP1 and APLP2) binding implicates Dab1 in pathways that regulate onset of the neurodegenerative disorder, Alzheimer's disease. LRP (lipoprotein receptor-related protein) mediates the endocytosis and clearance of APP-degraded beta-amyloid protein, the major component of amyloid plaques in Alzheimer's disease (Masters *et al.*, 1985; Kounnas *et al.*, 1995; Narita *et al.*, 1997). However, the exact biochemical and cellular functions of the Dab1-LRP and Dab1-APP interactions remain poorly understood. An indication of their possible roles may come from the study of two other APP-binding cytosolic adaptor proteins, X11 and Fe65, which have antagonistic effects in APP signaling. Overexpression of X11 increases APP half-life, while Fe65 overexpression increases APP proteolytic cleavage and formation of beta-amyloid protein (Borg *et al.*, 1996; Ando *et al.*, 2001). Transgenic mice overexpressing both Fe65 and APP form beta-amyloid plaques within six months of birth (Santiard-Baron *et al.*, 2005). It is interesting to note that hyperphosphorylation of the microtubule associated protein, Tau, is also implicated in the pathogenesis of Alzheimer's disease where it forms paired-helical filaments (Gotz *et al.*, 2004). Recent studies demonstrate a biochemical link between Tau and Fe65, regulated by Reelin-Dab1 pathway components Cdk5, Akt and GSK-3β (Iijima *et al.*, 2000; Barbato *et al.*, 2005; Ferrer *et al.*, 2005; Griffin *et al.*, 2005; Li *et al.*, 2005). Cdk5 is a kinase regulator of C-terminal Dab1, while Dab1 activates Akt kinase which down-regulates GSK-

3β activity, thereby reducing Tau phosphorylation and formation of Tau paired-helical filaments (Hiesberger *et al.*, 1999; Beffert *et al.*, 2002; Keshvara *et al.*, 2002; Ohkubo *et al.*, 2003). Like Dab1, Fe65 preferentially binds unphosphorylated APP-YENPTY docking sites (Trommsdorff *et al.*, 1998). Therefore, one can postulate that Dab1, when bound to APP, functions in a manner similar to that of either X11 or Fe65, by binding APP-YENPTY docking sites and modulating APP proteolytic cleavage. Likewise, Dab1 interactions with LRP may serve to modulate the rate of beta-amyloid protein degradation and beta-amyloid plaque formation. To further substantiate a link between Alzheimer's disease and the Reelin-Dab1 signaling pathway, analyses of mouse models for this disease show enhanced immunolocalization of Reelin, ApoER2, VLDLR, Dab1 and APP to neuritic beta-amyloid plaques (Li *et al.*, 2001; Wirths *et al.*, 2001; Weeber *et al.*, 2002; Grilli *et al.*, 2003; Motoi *et al.*, 2004). In humans, levels of Dab1-activating PtdIns4,5P_2 are also found to be reduced in the anterior temporal cortex of Alzheimer's patients (Stokes and Hawthorne, 1987). Furthermore, analysis of early cases of Alzheimer's disease reveals a dramatic reduction in the number of Reelin-producing Cajal-Retzius neurons (Riedel *et al.*, 2003; Baloyannis, 2005). Like Alzheimer's disease, formation of Tau paired-helical filaments is observed in *Reelin*$^{-/-}$, *ApoER2*$^{-/-}$*VLDLR*$^{-/-}$ and *Dab1*$^{-/-}$ mouse mutants (Hiesberger *et al.*, 1999; Brich *et al.*, 2003; Gong *et al.*, 2005; Iqbal *et al.*, 2005). Finally, levels of the Dab1 PI/PTB-interacting neuronal Wiskott-Aldrich syndrome protein (N-WASP) are significantly higher in the brains of Alzheimer's patients (Kitamura *et al.*, 2003). As discussed below, tyrosine phosphorylated N-

WASP promotes actin polymerization required for neurite extension (Suetsugu *et al.*, 2002). In Alzheimer's disease, N-WASP is implicated in the formation of abnormal dendritic neurites and aberrant neuronal sprouting, other hallmarks of this disease (Kitamura *et al.*, 2003).

1.3.7 The Dab1-PI/PTB interacts with N-WASP

The interaction between the Dab1 PI/PTB domain and N-WASP implicates Dab1 in agonist-induced regulation of actin polymerization (Suetsugu *et al.*, 2004). Cell migration requires the reorganization of the actin cytoskeleton (Miki and Takenawa, 2003). A migrating cell forms an extension in the direction of movement, called the cell leading edge. In neurons, the leading edge consist of growth cones at the tips of extending axons and dendrites. Filopodia and lamellipodia are critical mechano- and chemo-sensory components of growth cone extensions, and are involved in neuronal migration, and guidance and formation of inter-neuronal connections. Filopedia and lamellipodia form as a result of the dynamic rearrangement of the actin cytoskeleton. Regulation of actin polymerization normally involves a pathway comprised of cell division control protein 42 (Cdc42), N-WASP and the actin-related protein 2/3 (Arp2/3) complex. Cdc42 binds to the CRIB (Cdc42/Rac-interactive binding) motif within the N-WASP protein. This binding induces a conformational change in N-WASP by relieving an autoinhibitory interaction between the CRIB motif and the N-WASP C-terminus. This conformational change serves to activate N-WASP and promotes binding to and activation of Arp2/3, the actin polymerization nucleation core. The Dab1 PI/PTB domain binds to the non-canonical NRFY sequence located adjacent to the CRIB motif in N-WASP resulting in a similar conformational change observed upon Cdc42 binding resulting in the relaxation of N-WASP autoinhibition and activation of Arp2/3 independent of Cdc42 (Suetsugu *et al.*, 2004). Overexpression of Dab1 in non-neuronal cells induces filopodia and lamellipodia and this effect is dependent on the Dab1 PI/PTB domain. Expression of a Dab1 mutant which lacks this domain prevents formation of these actin dependent structures (Suetsugu *et al.*, 2004).

PtdIns4,5P$_2$ has also been shown to be a second messenger involved in actin-mediated intracellular vesicle trafficking and control of leading edge motility (Kanzaki *et al.*, 2004; Golub and Caroni, 2005). One of the effectors of PtdIns4,5P$_2$ is N-WASP. PtdIns4,5P$_2$-mediated signaling promotes Cdc42-independent associations between N-WASP and the actin polymerization nucleation core, Arp2/3. This increases actin assembly at the surface of endomembranes prior to intracellular vesicle transport (Benesch *et al.*, 2002). Thus, the identification of Dab1 as an N-WASP-interacting protein provides a mechanism for phospholipid-mediated induction of actin polymerization.

1.3.8 Potential for crosstalk in Dab1 and Dab2 signaling

The Dab1-PI/PTB domain has also been shown to bind to FxNPxY docking sites of the LDLR family member, megalin (LRP2), and that of a novel RasGAP protein called Dab2IP (Gotthardt *et al.*, 2000; Homayouni *et al.*, 2003). Megalin is an endocytic scavenger receptor involved in uptake and degradation of multiple intracellular ligands including apolipoproteins (B100, E, and J), lipoprotein lipase, lactoferrin, plasminogen (including their activators and activator inhibitors) and aprotinin (Orlando *et al.*, 1997; Morris *et al.*, 2002b). During development, megalin is expressed in neuroepithelia and inactivation of megalin leads to holoprosencephaly, a congenital disorder characterized by incomplete forebrain (prosencephalon) cleavage along the mid-sagittal axis required for the development of the right and left hemispheres (Spoelgen *et al.*, 2005). At maturity, megalin is enriched in renal proximal tubule cells (Nagai *et al.*, 2005). Megalin FxNPxY docking sites have also been shown to bind the Dab1 orthologue, Dab2, through the conserved PI/PTB domain (Oleinikov *et al.*, 2000). Co-expression of megalin and Dab2 are required for their mutual protein stability in endocytic vesicles, intracellular megalin transport, megalin-dependent uptake of intracellular ligands and Dab2-dependent activity (Morris *et al.*, 2002b; Nagai *et al.*, 2005).

Dab2-interacting protein (Dab2IP) was identified in a yeast two-hybrid screen for Dab2-interacting proteins (Wang *et al.*, 2002b). Like Dab1-Dab2IP, the Dab2-Dab2IP interaction occurs between the Dab2-PI/PTB domain and the Dab2IP-FQNPVY docking site. Dab2IP was first shown to stimulate Ras GTPase function *in vitro* and *in vivo* suggesting a role in the activation of the Ras pathway (Wang *et al.*, 2002b). Subsequent reports identified Dab2 and Dab2IP as tumour suppressors and their loss of function has been implicated in epithelial tumours (Dote *et al.*, 2004; Yano *et al.*, 2005; Dote *et al.*, 2005). The Dab1 interactions with megalin and Dab2IP implicate Dab2 in Reelin-mediated signaling. Formation of holoprosencephaly of the developing forebrain in *megalin*$^{-/-}$ mutant mice constitutes a yet-to-be-defined function for megalin and Dab2 in cortical development. Furthermore, the Dab1-Dab2 interrationship implicates Dab1 in epithelial function and in carcinogenesis. A role for Dab1 in cell types other than neurons is supported by the presence of Dab1 in non-neuronal tissues (Howell *et al.*, 1997a; Katyal and Godbout, 2004).

1.3.9 The Dab1 tyrosine phosphorylation domain transduces the Reelin signal

The central Dab1 tyrosine-rich domain contains four conserved SFK-dependent tyrosine phosphorylation sites: tyrosine-185 (Y^{185}QTI), tyrosine-198 (Y^{198}QYI), tyrosine-220 (Y^{220}QVP) and tyrosine-232 (Y^{232}DVP) (Howell *et al.*, 1997a). As mentioned earlier, Y^{185}QTI and Y^{198}QYI correspond to SFK phosphorylation sites (YQxI), while Y^{220}QVP and Y^{232}DVP correspond to the consensus Abl kinase recognition motif (YxVP) (Songyang *et al.*, 1993). These phosphorylation sites have been shown to be critical for Dab1 activation and protein turnover in Reelin-mediated cortical development (Howell *et al.*, 1999a). In support of this, Dab1 appears to be exclusively phosphorylated during embryonic development of the nervous system. Dab1 tyrosine phosphorylation is no longer detected once nerve tracts have been established (Howell *et al.*, 1997a). Mice expressing a mutant form of Dab1 (*Dab1*^{5F}) in which all five tyrosine residues have been converted to phenylalanine (F) display no detectable Dab1 tyrosine phosphorylation and show similar neuronal migration and cortical lamination defects as seen in *Reelin*$^{-/-}$, *Dab1*$^{-/-}$ or *ApoER2*$^{-/-}$*VLDLR*$^{-/-}$ mutant mice

(Howell *et al.*, 2000). Additional support for the importance of tyrosine phosphorylation in transducing the Reelin signal comes from the observation that mice that cannot transduce the Reelin signal (*reeler* or *ApoER2*$^{-/-}$*VLDLR*$^{-/-}$) or undergo Dab1 tyrosine phosphorylation (*Dab1*^{5F}), have increased levels of Dab1 protein compared to wild-type (Trommsdorff *et al.*, 1999; Howell *et al.*, 2000). This is likely due to the regulation of ubiquitin-mediated proteosomal degradation (Arnaud *et al.*, 2003a; Bock *et al.*, 2004; Suetsugu *et al.*, 2004), and will be discussed in section 1.3.16.

Reelin-dependent basal activation of Dab1 results in tyrosine phosphorylation and activation of SFKs (Bock and Herz, 2003). A study using SFK inhibitors and neuronal cell cultures deficient for three SFKs (Src, Fyn, Yes), identified Fyn as the main tyrosine kinase involved in Reelin-dependent Dab1 phosporylation (Arnaud *et al.*, 2003b). These investigators also found that specific inhibition of Src tyrosine kinase or reduction in Src levels results in only a minor reduction in Dab1 tyrosine phosphorylation, while inhibition or deficiency in Abl signaling has no effect on Dab1 tyrosine phosphorylation. Unexpectedly, *fyn*$^{-/-}$ mutant mice have only minor defects in cortical layer stratification, hippocampal neuronal positioning and do not display a *reeler*-like phenotype (Grant *et al.*, 1992; Yagi *et al.*, 1993; Yuasa *et al.*, 2004). The absence of a true *reeler* phenotype in these mice is likely due to a compensatory function exerted by Src and/or Yes kinases (Bock and Herz, 2003; Arnaud *et al.*, 2003b). In support of this, Dab1 can undergo tyrosine phosphorylation in *src*$^{-/-}$, *fyn*$^{-/-}$ or *yes*$^{-/-}$ single mutant mice (Howell *et al.*, 1997a; Ballif *et al.*, 2004). Furthermore, tyrosine phosphorylated Dab1 is able to pull-down Src and Yes kinases. An attempt to generate *src*$^{-/-}$*fyn*$^{-/-}$*yes*$^{-/-}$ triple knock-out mice yielded embryos that were not viable past embryonic day 9.5, thus precluding analysis of cortical development (Klinghoffer *et al.*, 1999). However, a recent study has shown that *src*$^{-/-}$*fyn*$^{-/-}$ double knock-out mice have some similarity to *Reelin*$^{-/-}$ and *Dab1*$^{-/-}$ mutant mice, with inverted layering of cortical neurons and defects in the migration of cerebellar Purkinje cells (Kuo *et al.*, 2005).

Dab1 phosphotyrosine mapping studies show that tyrosine-198 is the primary tyrosine residue to undergo Reelin-dependent phosphorylation (Keshvara *et al.*, 2001). Cultured *reeler* cortical ventricular zone neurons display no phosphotyrosine-198 immunoreactivity while stimulation of these cells with purified Reelin induces Dab1 tyrosine-198 phosphorylation (Magdaleno *et al.*, 2002). Tyrosine-220 was phosphorylated in Reelin-stimulated neurons, albeit to a lesser extent (Keshvara *et al.*, 2001). Reelin-induced basal activation of Dab1 and subsequent SFK-mediated activation is thought to potentiate (ie. hyperphosphorylate) Dab1 phosphorylation at tyrosines-198, 220 and 232 (Ballif *et al.*, 2004). This points to a possible hierarchy in Reelin-dependent Dab1 tyrosine phosphorylation, with phosphotyrosine-198 being the initial Dab1-activating event followed by phosphorylation of tyrosine-220 and tyrosine-232. This type of hierarchical/sequential phosphorylation is termed processive phosphorylation: phosphorylation of one SH2-binding site (tyrosine-198) allows for kinase recruitment and phosphorylation of another SH2-binding site (tyrosine-220 and -232) (Mayer *et al.*, 1995). Hyperphosphorylation primes Dab1 to form phosphotyrosine-specific associations with other SH2-containing proteins and activation of their corresponding signaling pathways (Fig. 1.11). Some of these SH2-containing proteins are in fact SH3-SH3-SH2 containing adaptor proteins, suggesting that Reelin-induced tyrosine phosphorylated Dab1 acts as a SFK-dependent scaffolding or recruitment factor in order to activate and synergize multiple signaling pathways. As discussed below, phosphotyrosine-198-specific interactions activate SFK-mediated pathways that may promote actin and microtubule polymerization associated with neuronal migration, while phosphotyrosine-220 and -232-specific interactions recruit adaptor proteins involved in actin depolymerization and inhibition of actin-mediated cell migration.

1.3.10 Dab1 phosphotyrosine-198-dependent interactions

In addition to SFKs, Dab1 phosphorylated at tyrosine-198 associates with phosphoinositol 3'-kinase (PI3K), involved in the activation of the PI3K/Akt/GSK-3β pathway (Fig. 1.12) (Beffert *et al.*, 2002; Ballif *et al.*, 2003; Bock *et al.*, 2003; Ohkubo *et al.*, 2003). PI3K typically functions as a heterodimer consisting of a regulatory subunit (p85α, p85β, p55γ) and a p110-catalytic subunit (α, β, δ) (Cantley, 2002). Association of the PI3K-p85α regulatory subunit with tyrosine phosphorylated Dab1 phosphorylates and activates the Akt serine/threonine kinase; however, p85α phosphorylation itself is not required. This would suggest that Dab1-mediated activation of PI3K occurs through a conformational change elicited by the interaction or, more intriguingly, through co-association with Dab1-bound PtdIns4,5P$_2$, previously shown to be an activator of PI3K (Kalesnikoff, Sly

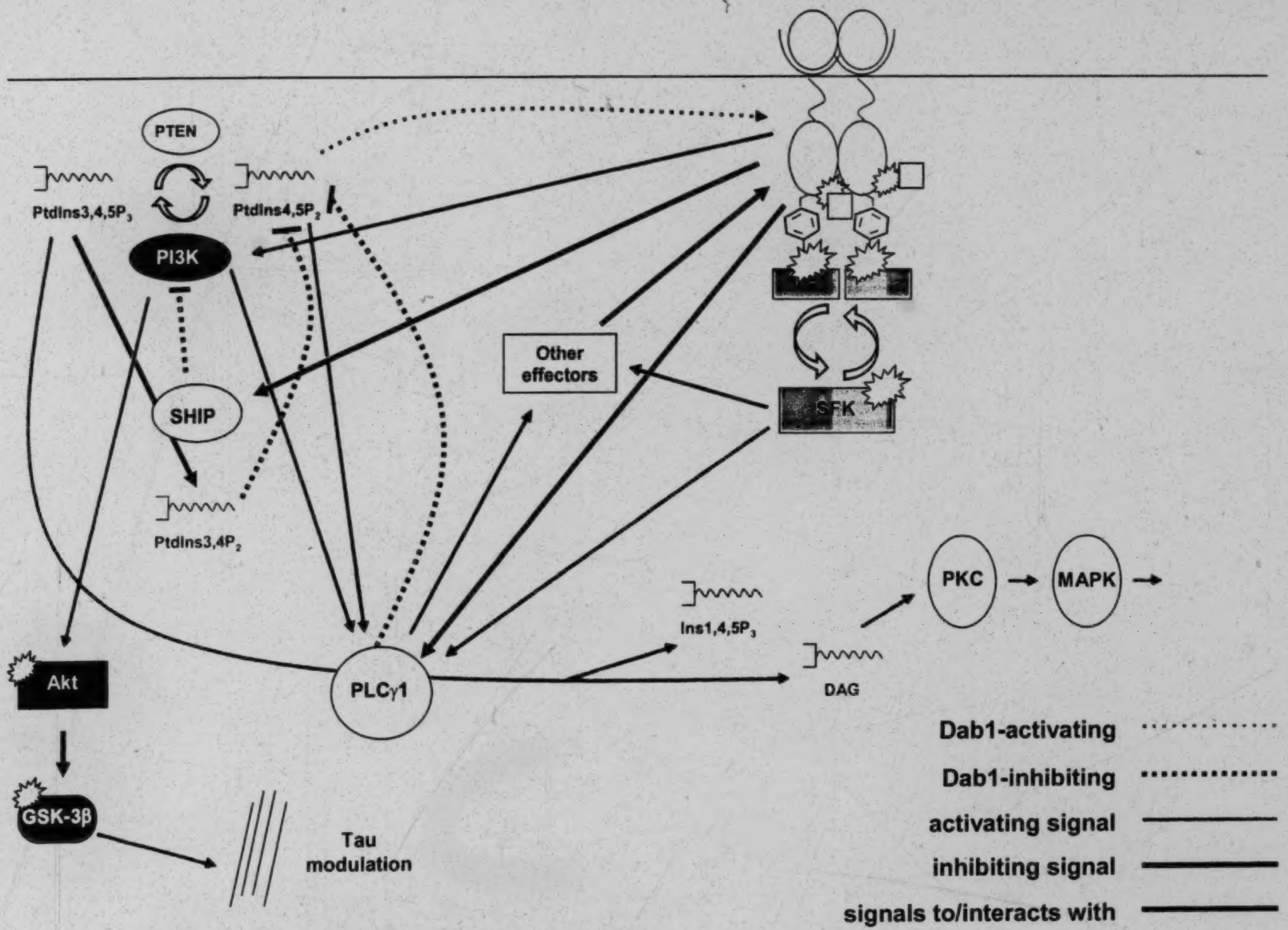

PTEN
PtdIns3,4,5P₃
PtdIns4,5P₂
PI3K
SHIP
PtdIns3,4P₂
Other effectors
SFK
Akt
GSK-3β
Tau modulation
PLCγ1
Ins1,4,5P₃
DAG
PKC
MAPK
Dab1-activating
Dab1-inhibiting
activating signal
inhibiting signal
signals to/interacts with

Fig. 1.12 - Dab1 phosphotyrosine-198-regulated pathways

et al. 2003). PI3K/Akt activation is SFK-dependent and not Abl-dependent as only SFK inhibitors prevent Akt phosphorylation in cultured neurons (Bock, Jossin et al. 2003). Akt kinase phosphorylates and down-regulates GSK-3β kinase activity resulting in reduced GSK-3β-mediated Tau phosphorylation. Tau is an axonal microtubule-stabilizing protein which regulates microtubule assembly and disassembly during neuronal migration (Mandelkow and Mandelkow, 1995). Regulation of Tau conformation and activity occurs through multiple phosphorylation sites (Avila *et al.*, 2004). While Tau undergoes steady-state levels of phosphorylation, neurodegenerative diseases associated with Tau hyperphosphorylation, such as Alzheimer's disease, result in the formation of neuritic Tau bundles termed paired-helical filaments (Brich *et al.*, 2003). Dab1/PI3K/Akt-mediated phosphorylation of GSK-3β reduces Tau phosphorylation thereby allowing Tau-microtubule interactions and microtubule-mediated neuronal migration (Ohkubo *et al.*, 2003). This effect is dependent on Reelin binding to the ApoER2 and VLDLR receptors (Beffert *et al.*, 2002). Primary neurons treated with Reelin-enriched medium display increased phosphorylation of Akt and GSK-3β while receptor blocking with RAP (receptor-associated protein) reduced these phosphorylation events. Furthermore, inhibition of PI3K and its associated pathway in cortical slice migration assays disrupts neuronal migration and Reelin-mediated formation of the cortical plate (Bock *et al.*, 2003). Lastly, in addition to their cortical neuronal migration defects, *Reelin-/-*, *VLDLR*$^{-/-}$*ApoER2*$^{-/-}$ and *Dab1*$^{-/-}$ mutant mice all display Tau hyperphosphorylation, thereby implicating the PI3K/Akt/GSK-3β pathway in Reelin-Dab1 signaling (Hiesberger *et al.*, 1999; Brich *et al.*, 2003).

The SH2-containing lipid metabolizing enzyme phospholipase C-gamma1 isoform (PLCγ1) has recently been shown to associate with tyrosine-198 phosphorylated Dab1 (Ballif *et al.*, 2004). The involvement of PLCγ1 in phospholipid metabolism and crosstalk with PI3K suggests a regulatory link between Dab1, PLCγ1 and the PI3K/Akt/GSK-3β pathway (Fig. 1.12). Activation of PLCγ1 results in enzymatic hydrolysis of the Dab1-activating phosphoinositide $PtdIns4,5P_2$ and reduction of intracellular $PtdIns4,5P_2$ levels (Kamat and Carpenter, 1997; Wang and Moran, 2002). This hydrolysis produces the second messengers inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$) and diacylglycerol (DAG) thereby activating the DAG/protein kinase C (PKC)/MAP kinase mitogenic pathway. PLCγ1 is regulated by the PI3K product, $PtdIns3,4,5P_3$ (Bae *et al.*, 1998). In turn, $PtdIns3,4,5P_3$ binds the pleckstrin homology domain of PLCγ1 thereby inducing its translocation and activation at the leading edge of migrating cells (Falasca *et al.*, 1998; Piccolo *et al.*, 2002). Tyrosine phosphorylated PLCγ1 is implicated in $PtdIns4,5P_2$-driven actin polymerization, membrane ruffling and beta-tubulin-mediated assembly of microtubules (Yu *et al.*, 1998; Chang *et al.*, 2005). $PtdIns3,4,5P_3$, when bound to PLCγ1, is hydrolyzed resulting in inhibition of PI3K and Akt kinase (Batty *et al.*, 1997). In conjunction with phosphoinositide-mediated activation, PLCγ1 is also activated by SFK-mediated tyrosine phosphorylation (Liao *et al.*, 1993; Marrero *et al.*, 1995). Furthermore, PLCγ1-mediated reduction of $PtdIns4,5P_2$ intracellular levels and SFK activation are jointly implicated in the phosphorylation and activation of Abl tyrosine kinase (Plattner *et al.*, 1999; Plattner *et al.*, 2003). SFK-induced Abl kinase activation is thought to involve Dab1 phosphotyrosines-220/232 and is implicated in Crk-dependent modulation of the actin cytoskeleton (Feller, 2001; Chen *et al.*, 2004). Phosphotyrosine-198-mediated activation of Abl provides further evidence of a hierarchical role for tyrosine-198 in processive phoshorylation of tyrosine-220 and tyrosine-232.

1.3.11 Dab1 phosphotyrosine-220/232 recruits Crk-family adaptor proteins

SFK-dependent phosphorylation of Dab1 at tyrosine-220 and tyrosine-232 allows for interactions with members of the Crk family of adaptor proteins including Nckα, Nckβ, CrkI, CrkII and CrkL (Feller, 2001; Pramatarova *et al.*, 2003; Ballif *et al.*, 2004; Chen *et al.*, 2004; Huang *et al.*, 2004). These SH3-SH3-SH2 domain-containing phosphoproteins link extracelluar cues with intracellular cytoskeletal remodeling events. Induction of Nck and Crk proteins are a result of their recruitment to the cell membrane through binding of their SH2 domain to tyrosine phosphorylated membrane-associated proteins (Lehmann *et al.*, 1990; Feller, 2001; Rivera *et al.*, 2004). At the same time, their SH3 domains allow for the recruitment and tyrosine phosphorylation of other proteins which regulate actin cytoskeletal dynamics and cell migration. Nck remodels the actin cytoskeleton primarily through its recruitment of N-WASP. Crk-mediated remodeling involves several protein regulators including C3G and the Rap1 and Rac GTPases (Feller, 2001). Nck and Crk play critical roles in actin-mediated cell migration. Fibroblast cell lines derived from *Nck*-deficient mice show defects in cell motility and organization of lamellipodia, while mouse embryonic fibroblasts (MEFs) derived from *CrkL*$^{-/-}$ mice show impaired integrin-mediated cell migration (Bladt *et al.*, 2003; Li *et al.*, 2003).

Nckβ has been found to associate with Dab1 (Pramatarova *et al.*, 2003; Huang *et al.*, 2004). Reelin stimulation of cultured neurons results in Dab1-dependent activation and redistribution of Nckβ to neuronal cell membranes. This effect is dependent on Dab1 phosphorylation at tyrosine-220 and tyrosine-232. In co-transfected rat fibroblast cells, Dab1 and Nckβ co-localize at the cell periphery while cells transfected with a *Dab1*^{5F} mutant do not (Pramatarova *et al.*, 2003). This indicates that Dab1 tyrosine phosphorylation is critical to the cellular localization of Nckβ. The Reelin-dependent interaction between Nckβ and Dab1 serves to disrupt actin filaments as the ends of Nckβ-enriched neuronal processes display a reduction in the formation of actin stress fibers (Pramatarova *et al.*, 2003). This study suggests that Reelin-induced activation of Nck inhibits neuronal migration by disrupting the actin cytoskeleton and restricting the extension of neuronal growth cones.

Crk proteins (CrkI, CrkII and CrkL) are derived from related genes, *Crk* (CrkI and CrkII) and *Crkol* (CrkL) (Ferrer *et al.*, 2005; Tang *et al.*, 2005). CrkI is a variant of CrkII and lacks the more C-terminal SH3 domain. Dab1 has recently been shown to interact with CrkI, CrkII and CrkL in a Reelin-induced SFK-dependent manner (Ballif *et al.*, 2004; Chen *et al.*, 2004; Huang *et al.*, 2004). Tyrosine→phenylalanine mutations at tyrosines 185, 198, 220, and 232 have revealed that the Dab1-Crk interactions are dependent on SFK-mediated phosphorylation of Dab1 at tyrosine-220 and tyrosine-232. Addition of PP2, a SFK inhibitor, ablates the Dab1-Crk interaction in neuronal cell cultures. Using a combination of GST-Dab1 affinity purification and co-immunorecipitation analysis, Chen *et. al.* (2004) have shown that the Dab1-CrkII/L interaction serves to mediate an indirect Dab1-Dock interaction. The authors suggest that a Dab1-Crk-Dock complex regulates Rac-dependent cell migration downstream of integrins. Rac is a small GTPase, which along with Rho and Cdc42, regulate actin polymerization. Activation of Dab1 induces redistribution of intracellular Crk resulting in its colocalization with Dab1 at the cell membrane. NBT-II cells co-transfected with Dab1 and Crk show reduced cell migration, thereby indicating that a consequence of Dab1-Crk interaction is interference with Crk-mediated cell migration through Rac-dependent alterations in the actin cytoskeleton. It has been postulated that this interference is caused by the inability of phosphorylated Crk to bind p130(CAS) (Crk-associated protein 130) (Li *et al.*, 2003). Dab1 tyrosine phosphorylation in Crk-expressing cells also results in loss of cell polarity, a lack of growth-cone associated lamellipodia and filopodia and a reduction in actin stress fiber formation (Chen *et al.*, 2004). Conversely, Crk-positive cells expressing the *Dab1*^{5F} mutant display normal cell migration and actin structural properties. This study shows that, like Nckβ, Reelin-induced recruitment of Crk proteins to tyrosine phosphorylated Dab1 induces actin depolymerization resulting in reduced cell migration (Pramatarova *et al.*, 2003; Chen *et al.*, 2004).

Another study by Ballif *et. al.* (2004) shows that Dab1-CrkL interaction mediates activation of C3G and Rap1, factors involved in the regulation of integrin-mediated cell adhesion and cell migration. However, these authors do not address how Dab1-mediated activation of this pathway might affect cell morphology, the actin cytoskeleton and cell migration. It should be noted that CrkII has recently been shown to inhibit N-WASP-mediated actin polymerization, thus reducing tensile strength in smooth muscle cells (Tang *et al.*, 2005). This points to the presence of a fine-tuning mechanism for CrkII in Reelin-mediated control of the actin cytoskeleton. The signal for actin polymerization induced by Dab1-N-WASP interactions may be opposed by phosphoDab1-activating CrkII interactions with N-WASP. Modulation of Dab1 tyrosine phosphorylation may shift this equilibrium in one direction or the other resulting in fine-regulation of actin polymerization and, consequently, the extent of cellular migration.

In *Drosophila*, Disabled was first identified as an Abl-interacting protein required for neuronal axonogensis (Gertler *et al.*, 1989). Despite the fact that mammalian Dab1 contains two conserved Abl-kinase recognition motifs (at tyrosine-220 and tyrosine-232), Reelin-mediated activation of Dab1 has primarily been shown to involve SFKs. Interestingly, SFK-dependent activation of Nck and Crk has been shown to recruit, phosphorylate and activate Abl tyrosine kinases, suggesting that Abl family kinases may be involved in Reelin-Dab1 signaling as downstream effectors of Nck and Crk adaptors (Smith *et al.*, 1999). Abl signaling is implicated in the regulation of cell migration. MEFs derived from $abl^{-/-}arg^{-/-}$ mice result in an association between Crk and p130(CAS) and display enhanced cell motility (Kain and Klemke, 2001). Introduction of inactive Abl in these cells does not decrease cell motility while reintroduction of active Abl does. This is a result of Abl phosphorylating Crk and abrogating its association with p130(CAS). The crucial importance of Abl in Crk-mediated actin dynamics provides strong evidence that SFK/Dab1-mediated activation and recruitment of Abl may be responsible for the cell migration inhibition observed in Dab1-Crk-dependent signaling (Pramatarova *et al.*, 2003; Ballif *et al.*, 2004).

1.3.12 Tyrosine phosphorylated Dab1 interacts with LIS1: involvement in microtubule-mediated transport

Mutation of the human lissencephaly gene (*LIS1*) (also known as *Pafah1b1*) is associated with the neurodegenerative disease Miller-Dieker Lissencephaly (Dobyns *et al.*, 1993; Reiner *et al.*, 1993). During embryonic cortical development *LIS1* is predominantly expressed in Reelin-expressing Cajal-Retzius cells (Clark *et al.*, 1997). Murine genetic interaction studies have shown that LIS1 is involved in the Reelin-Dab1 pathway (Assadi *et al.*, 2003). LIS1 associates with Dab1 in a Reelin-induced SFK-dependent manner. This Dab1 phosphorylation-dependent interaction is atypical compared to other Dab1 interactions in that LIS1 does not contain an SH2 domain. Furthermore, LIS1-Dab1 interaction is dependent on Dab1 phosphorylation at tyrosine-198 and tyrosine-220 and as such, is the only interaction documented to date requiring phosphorylation of these two residues. Mutation of either tyrosine site individually does not abrogate the Dab1-LIS1 interaction; however, mutation of both residues (Dab1$^{Y198F/Y220F}$) prevents this interaction. Furthermore, LIS1 proteins carrying mutations associated with the more severe forms of human lissencephaly show inability to form LIS1-Dab1 associations. LIS1 associates with the dynein microtubule motor protein and is involved in microtubule-dependent transport, neuronal migration and axonal outgrowth (Smith *et al.*, 2000). Inactivation of LIS1 slows down cellular migration in culture, while *LIS1*$^{-/-}$ mutant mice display aberrant radial glia, altered cortical neuron morphology and reduced neuronal cell migration during cortical development (Cahana *et al.*, 2001; Dujardin *et al.*, 2003).

1.3.13 The Dab1 C-terminus

Little is known regarding the role that the C-terminus of Dab1 plays in regulating Reelin-mediated signaling. However, there are a number of studies suggesting that the C-terminus acts to modulate transduction of the Reelin signal downstream from Dab1. For example, mice with a hypomorphic allele of Dab1, *Dab1*$^{p45/-}$ harboring only one *Dab1* allele encoding the Dab1^{271} isoform (which results in a C-terminal truncation), show no cerebellar defects and exhibit proper cortical lamination and neuronal positioning (Herrick and Cooper, 2002). While Reelin signaling is similar between Dab271- and Dab555-hemizygous mice, as evidenced by similar levels of Reelin-induced Dab1 tyrosine phosphorylation, only *Dab1*$^{p45/-}$ hemizygotes show a cell crowding phenomena within the marginal zone due to excessive migration of late-born neurons. This suggests that the C-terminus of Dab1 regulates neuronal positioning by mediating a type of stop-signal to prevent excessive neuronal migration. The exact mechanism is not known, although protein interaction domains within the Dab1 C-terminus may shed light on pathways that may be involved in the regulation of Reelin-mediated neuronal migration cues. The Dab1 C-terminus contains three key features: (i) serine-491 shown to be phosphorylated by Cdk5 serine/threonine kinase (Keshvara *et al.*, 2002), (ii) a myosin VI motor-binding domain (Morris *et al.*, 2002a), and (iii) a binding domain for SIAH-1A, an E3-ubiquitin ligase (Park *et al.*, 2003).

1.3.14 Regulation of Dab1 by Cdk5

Cdk5 has been shown to phosphorylate Dab1 on serine-491 *in vivo* (Keshvara *et al.*, 2001). This phosphorylation event occurs independently of Reelin signaling as Dab1 serine-491 phosphorylation is unchanged in *reeler* mice despite loss of Dab1 tyrosine phosphorylation. Cdk5- and Reelin-mediated signaling both regulate neuronal positioning but are thought to function through two independent, but parallel pathways. Mice deficient for Cdk5 or its protein regulators, p35 and p39, show similar but less severe cortical lamination and neuronal positioning defects as those observed in Reelin or Dab1 deficient mice (Ohshima *et al.*, 1996; Gilmore *et al.*, 1998; Kwon and Tsai, 1998; Ohshima *et al.*, 1999; Ko *et al.*, 2001). This functional overlap suggests that Dab1 may serve as a point of convergence for Cdk5- and Reelin-mediated signaling, mediated by tyrosine (Reelin) or serine (Cdk5) phosphorylation. This convergence would allow neuronal migration crosstalk, leading to the fine-tuning of neuronal positioning and cortical lamination during cortical development. Biochemically, cdk5-mediated phosphorylation of Dab1 serine-491 may serve as an allosteric regulator to facilitate conformational changes in Dab1 protein, thereby modulating Dab1 recruitment of SH2-containing proteins and adaptors involved in cytoskeletal regulation. Similar to Dab1, Cdk5 has been implicated as a regulator of processes involved in neurite outgrowth, cellular adhesion, LIS1-mediated axonal transport and the neuropathology of Alzheimer's disease (Nikolic *et al.*, 1996; Patrick *et al.*, 1999; Homayouni and Curran, 2000; Kwon *et al.*, 2000; Niethammer *et al.*, 2000; Sasaki *et al.*, 2000). Conversely, interactions between the Dab1 C-terminus and its protein-binding partners may occur directly with phosphoserine-491; however, of the known proteins that bind to the C-terminus of Dab1, none appear to be phosphoserine-491-specific.

1.3.15 Dab1, myosin VI and clathrin-coated vesicles: possible involvement in actin-mediated transport and endocytic trafficking

Clathrin-mediated endocytosis (Traub, 2003; Mousavi *et al.*, 2004) starts with localized site-specific recruitment of soluble clathrin and the heterotetrameric AP-2 adaptor protein complex to ligand-bound transmembrane receptors. Upon formation of a polygonic clathrin lattice, the clathrin-protein-membrane complex buds off internally to form an intracellular vesicle containing the internalized ligand-receptor. The AP-2 adaptor, when anchored by PtdIns4,5P_2 to the cell membrane, links the clathrin lattice with the internalized cargo. Cargo specificity, (e.g. for FxNPxY-containing ligand-receptor complexes) is mediated through the additional recruitment of PTB-containing/PtdIns4,5P_2-anchored endocytic accessory proteins. These accessory proteins form a complex with receptor cargo and clathrin through interactions with the AP-2 alpha appendage ear domain (alphaC-adaptin). Examples of FxNPxY-recognizing accessory proteins include Dab2, Numb and ARH (autosomal recessive hypercholesterolemia). Once these accessory proteins complete their primary function in the relaying of extracellular ligand-receptor binding signals, they perform their secondary function in targeting their signaling complex for clathrin-mediated cargo internalization and recycling. In FxNPxY-mediated endocytosis, vesicle formation is accompanied by association with myosin VI for minus-end directed transport along actin microfilaments away from the cell membrane (Buss *et al.*, 2001; Roberts *et al.*, 2004). These vesicles undergo endocytic trafficking and intracellular targeting to various subcellular targets including the plasma membrane for receptor redistribution, the leading edge of a migrating cell or to processing organelles such as the Golgi apparatus, endoplasmic reticulum or the proteosome (Buss *et al.*, 2002).

Interaction between Dab2 and myosin VI was originally discovered through Dab2 yeast two-hybrid studies and GST-pulldown assays (Morris *et al.*, 2002a). Analysis of the Dab2-myosin VI interaction led to the identification of a myosin VI binding domain in the Dab2 C-terminus [serine(S)680-tyrosine(Y)681-phenylalanine(F)682]. The same motif was subsequently identified within Dab1 [S445-Y446-F447], leading to the hypothesis that Dab1 also interacts with myosin VI. This domain was shown to be critical for pulldown of Dab1 using GST-myosin VI (Morris *et al.*, 2002a).

In Dab2-mediated signaling, the Dab2-myosin VI interaction facilitates clathrin-mediated receptor internalization and endocytic trafficking of FxNPxY-containing transmembrane receptors such as LDLR (Morris and Cooper, 2001; Mishra *et al.*, 2002; Morris *et al.*, 2002a). Dab2 forms a tripartite complex linking myosin VI with the clathrin adaptor AP-2. Dab2 interacts with the AP-2 alpha appendage ear region via duplicate aspartate(D)-proline(P)-phenylalanine(F) amino acid motifs located between the central region of Dab2 and the Dab2-myosin VI binding domain. Myosin VI is normally localized to membrane ruffles at the leading edge of cells and is only targeted for clathrin-mediated endocytosis when high levels of Dab2 protein are expressed (Dance *et al.*, 2004).

Multiple lines of evidence suggest that Dab1 is involved in myosinVI/clathrin-mediated endocytosis and intracellular trafficking of members of the Reelin-ApoER2/VLDLR complex. First, fluorescent immunolocalization studies of Dab1 in cultured neurons show similar punctate staining patterns as seen with Dab2, clathrin and AP-2 in NIH-3T3 cells (Howell *et al.*, 1999b; Morris and Cooper, 2001). Second, addition of Reelin in VLDLR-expressing cells results in Reelin-VLDLR complex internalization into endocytic vesicles (D'Arcangelo *et al.*, 1999). Third, Dab1 expression in cultured neurons enhances cell surface expression of VLDLR and the amount of cell-bound Reelin (Morimura *et al.*, 2005). *Dab1*-deficient neurons (*yotari*) show increased levels of VLDLR localized to the endoplasmic reticulum, suggesting that Dab1 is required for trafficking and exocytosis of VLDLR to the plasma membrane. Fourth, Dab1 colocalizes with internalized Reelin (Morimura *et al.*, 2005). Levels of Reelin vesicle internalization are 60% higher in wild-type cortical neurons than in *yotari* neurons. Furthermore, Reelin internalization is SFK-dependent as pharmacological inhibition of SFKs and Dab1 tyrosine phosphorylation in cultured neurons inhibits Reelin internalization.

1.3.16 Dab1 and ubiquination

In addition to relaying the Reelin signal intracellularly, tyrosine phosphorylation of Dab1 results in Dab1-ubiquination and targeting of Dab1 for proteosomal degradation. All models that mimic Reelin deficiency including mutant mice (*reeler*, *ApoER2*$^{-/-}$*VLDLR*$^{-/-}$, *Dab*^{5F} and *fyn*$^{-/-}$) and SFK inhibitors (PP2), show increased levels of Dab1 protein as a result of Dab1 hypophosphorylation (Arnaud *et al.*, 2003a; Bock *et al.*, 2004). Dab1 degradation also appears to be important in cortical plate formation as treatment of E13.5 cortical slices with the proteosomal inhibitor, epoxomicin, results in similar cortical plate defects as those seen in *reeler* mice. A recent study in which COS-7 cells were transfected with Fyn kinase and Dab1 indicates that tyrosine phosphorylated Dab1 undergoes ubiquitin-mediated proteosomal degradation in a Cbl-dependent manner (Suetsugu *et al.*, 2004). Cbl is an SH2-containing adaptor protein which functions to terminate protein tyrosine kinase signaling by substrate degradation. Cbl is an E3-ubiquitin ligase whose activity is mediated through its RING finger domain (Joazeiro *et al.*, 1999). Cbl recognizes tyrosine phosphorylated substrates through its SH2 domain resulting in substrate polyubiquination and targeting for proteosomal degradation. Cbl is widely expressed in the brain and is implicated in actin reorganization of growing neurites (Haglund *et al.*, 2004; Suetsugu *et al.*, 2004).

Dab1 is also implicated in regulating the activity of SIAH-1A, another E3-ubiquitin ligase (Park *et al.*, 2003). Through yeast two-hybrid analysis, co-immunoprecipitation and binding studies, the Dab1 C-terminus was found to interact with SIAH-1A. This interaction has been shown to inhibit SIAH-1A self-ubiquination and substrate ubiquination activities. Dab1-SIAH-1A interaction may therefore prevent degradation of substrates implicated in neuronal development. For example, SIAH-1 binds, ubiquinates and targets the PTB-containing protein Numb for proteosomal degradation (Susini *et al.*, 2001). Numb is involved in the regulation of proliferation and differentiation in the retina (Verdi *et al.*, 1999; Susini *et al.*, 2001). Numb functions as a suppressor of Notch, a receptor protein that promotes retinal gliogenesis and inhibits neurogenesis (Zhong *et al.*, 1996; Wakamatsu *et al.*, 1999). Dab1-mediated inhibition of SIAH-1 E3-ligase activity may be a way to stabilize Numb and suppress Notch thereby promoting neuroretinal development.

1.3.17 Evidence for opposing Reelin-Dab1 pathways in the regulation of neuronal migration

In addition to ApoER2 and VLDLR, transduction of the Reelin signal to Dab1 has been shown to occur through alpha3beta1 integrins. Reelin binds alpha3beta1 integrin through a section of Reelin that is N-terminal in location to the region that binds ApoER2 and VLDLR. Furthermore, Dab1 has recently been shown to interact with cytoplasmic alpha3beta1 integrin, and alpha3beta1 integrin-expressing cells also show modulation of Src and Fyn-tyrosine kinase activities (Schlaepfer and Hunter, 1998; Sato *et al.*, 1999; Zhang *et al.*, 2003; Sanada *et al.*, 2004; Schmid *et al.*, 2005). These results show a strong link between alpha3beta1 integrins and the Reelin-Dab1 signaling pathway. However, in contrast to ApoER2/VLDLR mediation of Reelin-Dab1 signaling, the function of alpha3beta1 integrin-mediated Reelin signaling is to inhibit neuronal cell migration and promote neuronal cell detachment from radial glial cells (Dulabon *et al.*, 2000). Reelin-mediated neuronal detachment also requires that Dab1 be phosphorylated at two tyrosine phosphorylation motifs, tyrosine(Y)-220 and Y232. (Forster *et al.*, 2002; Hartfuss *et al.*, 2003). These motifs have been identified as Abl family kinase/Crk recognition sites [tyrosine(Y)-X-valine(V)-proline(P)] (Howell *et al.*, 1997b). Newborn neurons within the cortex of *scrambler* (*Dab1*$^{-/-}$) mice show abnormal adhesion to radial glia processes throughout the entire course of neuronal migration and fail to detach in a timely manner (Sanada *et al.*, 2004). In contrast, cortical neurons of normal embryos detach from radial glial processes at late stages of migration. "Knock-in" of mutant *Dab1*, harboring tyrosine(Y)-220→phenylalanine(F)-220 or Y232F substitutions, into *scrambler* mice fails to rescue this migration defect while knock-in of the *Dab1*^{Y198F} mutant does. These observations provide strong supportive evidence that Reelin-Dab1 signaling through ApoER2/VLDLR or alpha3beta1 result in opposing effects, with ApoER2/VLDLR-mediated Reelin signal transduction promoting neuronal migration and alpha3beta1 integrin-mediated Reelin signal transduction inhibiting migration and promoting neuronal detachment from the radial glial scaffold. As two different regions of Reelin are involved in the binding of ApoER2/VLDLR receptors and alpha3beta1 integrins, Reelin may be able to bind and cluster both types of receptors simultaneously in order to activate a type of synergized antagonistic neuronal migration signal. In this model, signaling through both types of Reelin receptors could be a mechanism for fine-tuning neuronal-glial cell adhesion and for regulating the extent of neuronal migration during cortical development. In support of this model, Reelin, alpha3beta1 integrin, ApoER2 and VLDLR have all been shown to co-immunoprecipitate from brain homogenates, thereby linking these molecules in a common complex *in vivo* (Dulabon *et al.*, 2000). Furthermore, cortical neurons derived from mice deficient in alpha3beta1 integrin did not display inhibition of neuronal migration upon Reelin treatment (suggesting an inability to respond to Reelin) and showed reduced detachment from radial glial cells (Dulabon *et al.*, 2000). Alpha3-integrin (-/-) mutant mice also displayed defects in neuronal migration and cortical lamination, while conditional inactivation of beta1-integrin in cortical neurons and glia disrupted cortical layering (Anton *et al.*, 1999; Graus-Porta *et al.*, 2001; Schmid *et al.*, 2004; McCarty *et al.*, 2005).

1.3.18 The Reelin-Dab1 signaling pathway in the retina

The retina is considered to be an extension of the brain and contains the same cell types found in brain: neurons and glia. Like the brain, the retina is a highly ordered laminated structure characterized by migration of retinal neurons, their positioning into specific layers and the formation of synaptic circuitry through interneuronal connections between outgrowing dendrites and axons. As expected, many pathways that govern neuronal development are common between the brain and the retina.

Reelin is expressed in the retinal ganglion cell layer of a variety of species (Schiffmann *et al.*, 1997; Bernier *et al.*, 1999; Goffinet *et al.*, 1999; Bernier *et al.*, 2000). In humans and cats, Dab1 is expressed in different retinal cell types, while Dab1 appears restricted to a subset of amacrine cells called glycinergic AII, in rodents, guinea pigs and rabbits (Rice and Curran, 2000; Rice *et al.*, 2001; Lee *et al.*, 2004). Furthermore, both Reelin and Dab1 protein are detected in the inner plexiform layer (IPL), the region where interneuronal connections between the amacrine and ganglion cells occur. Other components of the Reelin-Dab1 signaling pathway expressed during vertebrate retinal development including Src, Fyn, PI3K, Tau, APP, PLCγ1, myosin VI and Rac/Rho (Vardimon *et al.*, 1991; Ingraham *et al.*, 1992; Loffler *et al.*, 1995; Worley and Holt, 1996; Peng *et al.*, 1997; Breckler *et al.*, 2000; Wong *et al.*, 2000; Pimentel *et al.*, 2002).

Ultrastructural analysis of *reeler* and *scrambler* retinas reveal a number of amacrine cell abnormalities common to both mutant mice. These include reduced amacrine cell dendrite density and altered organization and layering of amacrine cell processes within the IPL (Rice and Curran, 2000). Furthermore, these mice show a reduction in the number of rod bipolar cells within the INL. Electroretinograph analyses of *reeler* and *scrambler* mice reveal attenuated responses to visual stimulation due to disruption of the rod photoreceptor-driven pathway (Rice *et al.*, 2001). The combined expression of Reelin, Dab1 and associated proteins in the retina along with mouse knock-out data provide strong evidence that the Reeln-Dab1 signaling pathway is involved in the growth, differentiation and synaptic organization of the vertebrate retina.

1.4 RESEARCH OBJECTIVES

1.4.1 **Chapter 2.** *Alternative splicing modulates Disabled-1 (Dab1) function in the developing chick retina*

The Reelin-Disabled 1 (Dab1) signaling pathway plays a critical role in neuronal cell positioning in the brain. We have isolated two alternatively spliced variants of Dab1 from chick retina, an early form (chDab1-E) expressed in undifferentiated cells and a late form (chDab1-L) expressed in amacrine and ganglion cells. A key difference between the two forms is the exclusion in chDab1-E of two Src-related tyrosine kinase recognition sites implicated in Reelin-mediated Dab1 tyrosine phosphorylation. Retinal cultures transfected with a chDab1-L expression construct undergo a dramatic change in morphology, accompanied by formation of numerous thin elongated processes, increased tyrosine phosphorylation, activation of Src family kinase(s), and increased levels of the axonal outgrowth protein GAP-43. In contrast, chDab1-E transfectants retain an undifferentiated morphology. Mutational analysis implicates a specific tyrosine (tyr-198) in the morphological and biochemical alterations associated with chDab1-L expression. We propose that alternative splicing of chDab1 represents an effective and flexible way of regulating the Reelin-Dab1 signaling pathway in a mixed cell population, by ensuring that secreted Reelin activates the signaling cascade only in target neuronal cells.

1.4.2 **Chapter 3.** *Disabled-1 is alternatively spliced in human retina and tumours of neuroectodermal lineage*

To address whether Dab1-E and Dab1-L isoforms exist in species other than chicken, we characterize Dab1 expression in the human fetal retina. In this chapter, we show that the same two Dab1 isoforms identified in chicken also exist in human retina. Transfection of huDab1-E and huDab1-L in chick retina cultures generates the same morphology and properties observed with the chicken isoforms, suggesting evolutionarily conserved roles for Dab1-E and Dab1-L in vertebrates. In the second part of this chapter, we examine Dab1 alternative splicing in retinoblastoma and neuroblastoma, tumours derived from cells of neuroectodermal origin. Our data indicate that Dab1 alternative splicing is deregulated in retinoblastoma and neuroblastoma cell lines and tumours, and that the Reelin-Dab1 pathway is likely non-functional in these tumour cells.

1.4.3 Chapter 4. *Molecular analysis of Dab1 tyrosine phosphorylation and protein interactions*

In this chapter, we characterize the contribution of four conserved tyrosine residues, encompassed within two SFK (tyrosine-185 and tyrosine-198) and two Abl kinase/Crk (tyrosine-220 and tyrosine-232) recognition motifs, to Reelin-mediated Dab1 tyrosine phosphorylation and activation. We use an *in vitro* mutagenesis approach to generate GFP-Dab1-L expression constructs containing combinations of single, double and triple tyrosine→phenylalanine substitutions. Biochemical analysis of primary chick retinal cells transfected with these constructs reveals a hierarchical relationship between these four tyrosines in Reelin-mediated Dab1 phosphorylation and activation of Src-family kinases.

SHP-2, a tyrosine phosphatase, has been previously shown to associate with Dab1. Using SHP-2 and GFP-Dab1-L expression constructs, we examine the role of SHP-2 in Reelin-Dab1 signaling. To identify proteins that associate with specific Dab1 isoforms, we used the yeast two-hybrid technique to screen huDab1-E and huDab1-L bait proteins with a human fetal brain cDNA expression library. Although our screen did not yield Dab1 isoform-specific interactions, we have identified a number of novel Dab1-protein interactions associated with actin- and microtubule-mediated transport and protein degradation pathways.

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Chapter 2

Alternative splicing modulates Disabled-1 (Dab1) function in the developing chick retina

Running title: *chDab1* alternative splicing in the chick retina

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2.1 INTRODUCTION

Transmission of the visual signal to the brain depends on the proper organization of differentiated retinal cells into distinct nuclear and synaptic layers. Photoreceptors, which capture light photons, are located in the outer nuclear layer closest to the retinal pigmented epithelium. These cells transmit a neural signal via the outer plexiform layer to the inner nuclear layer where the input signal is further processed by four classes of neuronal cells (amacrine, bipolar, horizontal, interplexiform). The visual signal is then transmitted to the innermost nuclear layer of the retina, called ganglion cell layer, via the inner plexiform layer. Ganglion cells send their axons through the optic nerve to form connections with specific targets in the brain, resulting in the projection of the visual image to the brain (Dowling, 1987). These six classes of neuronal cells as well as the Müller glial cells, located in the inner nuclear layer, are derived from neuroectodermal precursor cells called retinoblasts which migrate to their proper location as they differentiate (Turner et al., 1990).

The Reelin-Dab1 signaling pathway has been implicated in the positioning of migrating neurons in the brain (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Howell *et al.*, 1997b). Reelin is a secreted extracellular matrix glycoprotein that binds to the very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) (D'Arcangelo *et al.*, 1999; Trommsdorff *et al.*, 1999). Dab1, an intracellular adapter protein, binds to NPxY motifs located in the cytoplasmic tails of Reelin receptors. Mice deficient in Reelin (*reeler*), Dab1 (*yotari/scrambler*) or both VLDLR and ApoER2 have similar neuronal cell positioning defects in the brain, with inversion of neuronal layers in the cerebral cortex, and laminar defects in the cerebellum and hippocampus (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Trommsdorff *et al.*, 1999).

Binding of Reelin to its receptors induces Dab1 tyrosine phosphorylation and stimulates the activation of Src family tyrosine kinases and Akt serine/threonine kinase (Howell *et al.*, 1999; Beffert *et al.*, 2002; Ballif *et al.*, 2003; Benhayon *et al.*, 2003). Src, Fyn and Yes kinases are involved in Dab1 phosphorylation while phosphorylated Dab1 enhances their activity, suggesting a self-regulating tyrosine signaling cascade (Arnaud *et al.*, 2003; Bock and Herz, 2003). A possible downstream effector of phosphorylated Dab1 is glycogen synthase kinase 3β (GSK-3β) which is downregulated in response to Reelin signalling (Beffert *et al.*, 2002; Ohkubo *et al.*, 2003). GSK-3β modulates the activity of the microtubule-associated protein Tau involved in microtubule assembly during neuronal differentiation (Ishiguro et al., 1993).

The main form of mammalian Dab1 has an open reading frame of 555 amino acids and consists of an N-terminal domain that associates with Reelin receptors, an internal domain containing Reelin-dependent tyrosine phosphorylation sites and a C-terminal domain implicated in the modulation of Reelin-Dab1 signaling (Herrick and Cooper, 2002). The human and mouse *Dab1* genes span 1100 kb and have 14 main coding exons. Alternative internal and 5' exons have been identified (Bar *et al.*, 2003) and different-size isoforms have been detected by western blot analysis. Some Dab1 isoforms show developmental- and tissue-specific expression patterns suggesting a role in embryogenesis and organogenesis.

We have isolated chicken Dab1 (chDab1) cDNA from undifferentiated retinal cells using a differential display strategy. Here, we show that chDab1 undergoes alternative splicing as a function of developmental stage, with early and late forms of the protein expressed in proliferating and differentiating cells, respectively. Transfection of primary retinal cultures with expression constructs containing either the early or the late form of chDab1 results in dramatic changes in cellular morphology, with cells transfected with the late form producing numerous thin and extended processes. This morphological alteration is accompanied by Src family kinase activation and is dependent on tyrosine phosphorylation sites that are spliced out in the early form of chDab1. We propose that the absence of these tyrosine phosphorylation sites in the early form of chDab1 results in uncoupling of the Reelin-Dab1 pathway.

2.2 MATERIALS AND METHODS

2.2.1 DD-PCR analysis

DD-PCR analysis was performed as described (Liang and Pardee, 1992; Godbout and Andison, 1996). Poly(A)$^+$ RNA was extracted from chick retinas at ED3.5, ED5 and ED16, and from chick brains at ED5 and ED16. The cDNAs were generated by reverse transcription of 1 μg poly(A)$^+$ RNA using T_{11}MN primers (where M represents G, A or C and N represents any nucleotide). The cDNAs were PCR-amplified in the presence of ^{35}S-dATP with Taq polymerase

(GE Healthcare) using a $T_{11}MN$ primer and a random decamer oligonucleotide primer (OPA) (Operon Biotechnology Inc.). Amplified cDNAs were electrophoresed on a 6% polyacrylamide-urea gel and visualized by autoradiography. Selected bands were then re-amplified and ligated into a pBluescript vector (Stratagene) with a T overhang at the *Eco*RV site.

2.2.2 Screening of an ED7 chick retina cDNA library

The cDNA library was prepared as previously described (Godbout, 1993) except that the cDNA was produced from ED7 poly(A)$^+$ RNA. Approximately 2 X 10^5 bacteriophage were filter-lifted and hybridized with the DD-PCR-isolated 532 bp *chDab1* cDNA fragment. Purified clones were obtained after three rounds of screening. An ABI 310 automated sequencer was used for sequencing of the cDNAs using a combination of sequential deletion and sequence specific oligomers.

2.2.3 Northern blot analysis

Poly(A)$^+$ RNA was isolated from retina, brain, heart, liver, kidney and gut at the developmental stages indicated in the Fig. 2.1 legend. Two µg of each poly(A)$^+$ RNA, were electrophoresed in a 6% formaldehyde-1.5% agarose gel in MOPS buffer and transferred to nitrocellulose. The 532 bp chDab1 band, obtained using $T_{11}MG$ and OPA-10 (5'-GTGATCGCAG-3'), was used to probe the blot. Filters were washed at 55°C in 0.1X SSC, 0.1% SDS and visualized by autoradiography. Hybridization to mouse actin cDNA was used as a control for mRNA level variation.

2.2.4 Western blot analysis

Whole cell lysates were electrophoresed in a 10% polyacrylamide-SDS gel followed by electroblotting onto nitrocellulose. Blots were incubated with either rabbit anti-Dab1 antibody (1:5000 dilution) (Rockland Immunochemicals), goat anti-actin antibody (I-19) (1:500) (Santa Cruz Biotechnology), mouse anti-phosphotyrosine antibody (P-Tyr-1000) (1:1000) (Cell Signaling Technologies), mouse anti-GAP-43 antibody (GAP-7B10) (1:500) (Sigma) or mouse anti-GSK-3β antibody (1:2500) (BD Biosciences). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) using the ECL detection system (GE Healthcare).

2.2.5 RT-PCR analysis

One μg poly(A)$^+$ RNA from retina (ED5, 7, 10, 16) and brain (ED3.5, 5, 16) was reverse transcribed using an oligo d(T) primer and Superscript reverse transcriptase (Invitrogen). PCR was carried out using 1/20 of the cDNA generated. Primer set P1 and P2 was used for the analysis of the deletion region, primer set P3 and P4 was used for the analysis of the insertion region, and primer set P1 and P4 was used for the analysis of both the deletion and insertion region (Fig. 2.2). DNA fragments were run in 10% (P1/P2 and P3/P4 primer sets) and 15% (P1/P4 primer set) polyacrylamide gels. For sequencing, amplified DNAs were cloned into the pBluescript-T overhang vector as described under "DD-PCR Analysis".

2.2.6 In Situ hybridization

Digoxigenin (DIG)-labeled sense and antisense riboprobes were generated by *in vitro* transcription of linearized plasmids using T3 or T7 polymerase (Roche). Riboprobes were prepared from the 532 bp *chDab1* cDNA fragment isolated by DD-PCR, a 77 bp cDNA generated by PCR amplification of *chDab1-E* using primer set P7 and P8 flanking the insertion region (Fig. 2.2), a 147 bp cDNA fragment generated by PCR amplification of *chDab1-L* using primer set P9 and P10 flanking the deletion region (Fig. 2.2), a 2.0 kb carbonic anhydrase II (*CA-II*) cDNA fragment (Godbout, 1993) and a 1.2 kb *AP-2β* cDNA fragment (Bisgrove and Godbout, 1999). Tissues were fixed in 4% PBS-buffered paraformaldehyde at 4°C, cryoprotected with 12%, 16% and 18% sucrose and embedded in O.C.T. (Tissue-Tek, Miles Inc., Elkhart, IN). Frozen sections (6-8 µm) were prehybridized at 50°C-55°C in 40% formamide, 10% dextran sulphate, 1X Denhardt's solution, 4X SSC, 10 mM DTT, 1 mg/ml yeast tRNA and 1 mg/ml heat denatured herring testis sperm DNA. Riboprobes were heat-denatured and hybridized to tissue sections overnight at 50°C-55°C. Tissue sections were washed as described (Belecky-Adams et al., 1997) and incubated with alkaline-phosphatase (AP)-conjugated anti-DIG antibody. The signal was detected with BCIP/NBT after polyvinyl alcohol enhancement (Jowett, 1997).

2.2.7 Transfection analysis

For construction of GFP-fusion constructs, full-length *chDab1-E and -L* cDNAs were generated by RT-PCR using poly(A)$^+$ RNAs from ED5 and ED16 chick retinas, respectively. The cDNAs were amplified with a mixture of *Taq/Pfu* polymerase (100:1) using the P5/P6 primer set spanning the entire open reading frame. *Bam*HI and *Eco*RI restriction endonuclease sites were added to the ends of the oligonucleotides to allow in frame cloning. cDNAs were purified, digested with *Eco*RI and *Bam*HI and cloned into the pEGFP-C1 vector (Clontech). Both cDNAs were sequenced to ensure that they were error-free. Primary retinal cultures were prepared from ED5 chick retinas trypsinized prior to plating onto glass coverslips (one-twelfth of a retina per 12 mm coverslip). Cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum and incubated in a 5% CO_2 humidified chamber. Cells were transfected by calcium phosphate DNA precipitation and the DNA removed after 16 hrs. Thirty hrs later, the cells adhering to coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 mins and permeabilized for 5 mins in 0.5% Triton X-100/PBS. Cells were incubated overnight with mouse anti-phosphotyrosine antibodies [PT-66 (1:250) (Sigma), P-Tyr-1000 (1:250) (Cell Signaling Technologies)], anti-phospho-Src$^{(Y416)}$ (9A6) (1:50) (Upstate), or anti-GAP-43 antibody (GAP-7B10) (1:250) (Sigma) followed by Alexa 555 goat anti-mouse secondary antibody (Molecular Probes) (1:200) for one hr. The coverslips were mounted on slides using glycerol containing 1 mg/ml *p*-phenylenediamine +

1 µg/ml 4', 6'-diamidino-2-phenylindole (DAPI). Cells were viewed on a Zeiss LSM 510 confocal microscope.

For Reelin inhibition experiments, Dab1-L-tranfected retinal cells were treated with CR-50 antibody (kindly provided by Dr. T. Curran, St. Jude Children's Research Hospital, TN). After the removal of DNA from transfected cells, 1 ul of CR-50 antibody was added to each well (coverslip) for 24 hours. After a second round of CR-50 antibody treatment, cells were fixed and stained with anti-phospho-SFK antibody.

2.2.8 Site-directed mutation analysis

Site directed mutagenesis of chDab1-L tyr-185, tyr-198, tyr-200, tyr-220 and tyr-185/tyr-198 was carried out by sequential PCR (Cormack and Castano, 2002). Partially complementary primers containing a point mutation corresponding to a tyr → phe substitution [TA(T/C) → TT(T/C)] were used in conjunction with pEGFP-C1 vector primers located upstream of the *Eco*RI site and downstream of the *Bam*HI site to generate DNA fragments corresponding to full-length chDab1-L, each mutated at a specific tyrosine residue. DNA fragments were annealed, extended and amplified using pEGFP-C1 vector primers. The DNA was digested with *Eco*RI and *Bam*HI and cloned into pEGFP-C1. Constructs were sequenced to ensure that they were error-free. Expression of full-length GFP-chDab1-L mutant proteins was confirmed by transfection and western blot analysis.

2.2.9 Immunofluorescence of retinal sections

Retinal tissue sections were prepared as described under "*In situ* hybridization". Frozen sections were rehydrated in PBS, fixed in 4% PBS-buffered paraformaldehyde and permeabilized in 1% PBS-buffered NP-40. Sections were double-stained with rabbit polyclonal anti-Dab1 (1:500) and either mouse anti-phosphotyrosine (1:500), anti-phospho-Src$^{(Y416)}$ (1:25), or anti-GAP-43 (1:250) antibodies, followed by fluorescent secondary antibodies (Alexa 555 goat anti-rabbit and Alexa 488 goat anti-mouse) (1:150). Sections were counterstained with the Hoescht 33258 fluorescent nuclear stain (Molecular Probes) and mounted with Fluorosave (Calbiochem). Images were collected with a Zeiss-Axioplan II microscope (Carl Zeiss) equipped with a cooled charge-coupled device camera (Cooke Corporation).

2.3 RESULTS

2.3.1 Dab1 cDNA is highly expressed in chick retina compared to brain

To identify genes enriched in the undifferentiated chick retina, differential display (DD)-PCR analysis was carried out with poly(A)$^+$ RNA from retina at embryonic day (ED) 3.5, ED5, ED16 and brain at ED5 and ED16. Using primer pair T_{11}MG and OPA-10, a 532 bp band was generated that was more intense in ED3.5 and ED5 retina than in ED16 retina and brain. Sequencing of this cDNA revealed a high degree of similarity to the 5' ends of human and mouse *Dab1*. The 532 bp cDNA was used to probe a northern blot of retina, brain, heart, liver, kidney and gut at different developmental stages. Highest levels of *chDab1* RNA were found in ED5 and ED10 retina as well as ED16 kidney, with ~2-3-fold lower levels in ED16 retina (Fig. 2.1A). Considerably lower levels of *chDab1* RNA were found in brain, heart, liver and gut.

Dab1 protein was examined by western blot analysis of whole cell lysates prepared from ED4, ED7 and ED16 retina. The pattern of expression was similar to that observed by northern blotting, with higher levels of chDab1 protein in ED4 and ED7 retina compared to ED16 retina (Fig. 2.1B). In addition to quantitative changes, differences were observed in the banding patterns of ED4, ED7 and ED16 retina, with the lowest band disappearing by ED16. The number of bands observed in each lane suggest the presence of multiple isoforms and/or different post-translational modifications.

2.3.2 Developmentally-regulated alternative splicing of chDab1

Full-length *chDab1* cDNA encoding a predicted open reading frame of 535 aa was obtained by screening an ED7 chick retina cDNA library with the 532 bp DD-PCR DNA fragment (Fig. 2.2). Overall, the chicken Dab1 protein sequence had a high level of similarity to the 555 aa human and mouse sequences (92% identical; 96% similar), with two major differences: (i) a deletion of 105 bp (35 aa) located immediately after aa 186 and corresponding to aa 187-221 of human/mouse Dab1, and (ii) an insertion of 57 bp (19 aa) located after aa 206. Based on the exon/intron structure of human *Dab1* genomic DNA, the 105 bp deletion region corresponds to two exons (exons 7 and 8) and the 57 bp insertion region corresponds to a single exon (exon 9-2) (Bar *et al.*, 2003). While

Fig. 2.1 - Northern and western blot analyses of chDab1 expression. (**A**) Northern blots were prepared using poly(A)$^+$ RNA (2 μg/lane) extracted from retina (ED5, ED10, ED16), brain (ED5, 10, 16), heart (ED5, 15), liver (ED5, 10, 15), kidney (ED7, 16) and gut (ED16). The filter was sequentially hybridized with ^{32}P-labeled: (i) 532 bp *chDab1* cDNA and (ii) actin cDNA. The extra bands obtained with the actin probe in heart and gut represent tissue-specific actin mRNAs. We consistently find actin RNA to be low in liver, especially at later developmental stages. (**B**) Western blots were prepared using ED4, ED7 and ED16 total chick retina extracts (50 μg protein/lane). The filter was sequentially incubated with rabbit anti-Dab1 antibody (1:5000) and goat anti-actin antibody (1:200). Molecular mass standards (in kDa) are indicated on the left.

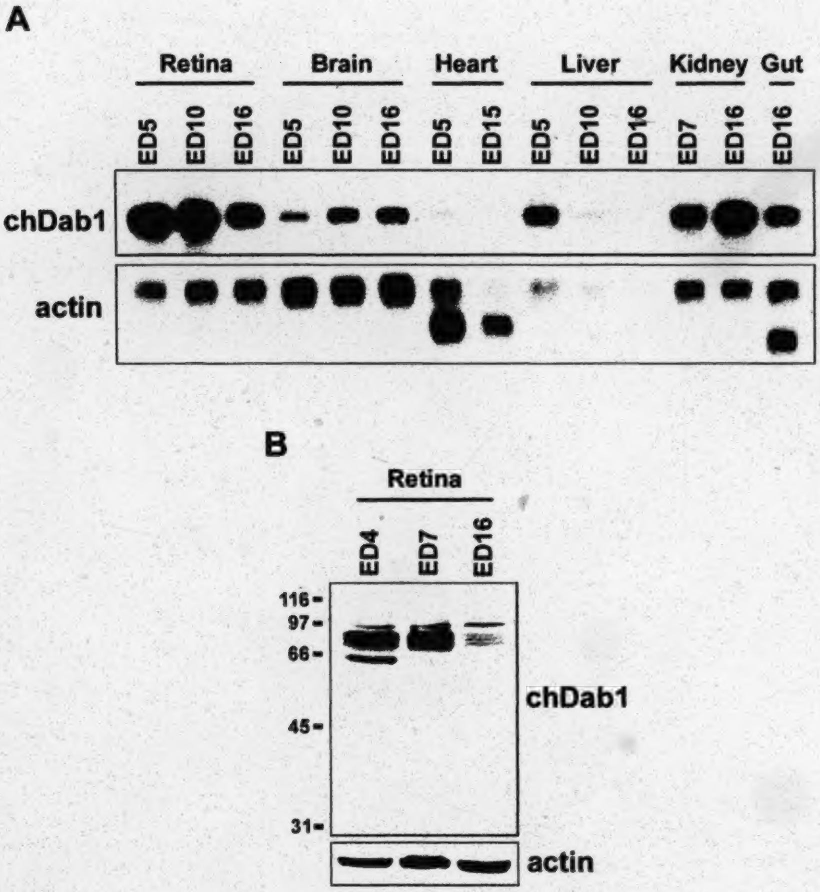

A
Retina
Brain
Heart
Liver
Kidney
Gut
ED5
ED10
ED16
ED5
ED10
ED16
ED5
ED15
ED5
ED10
ED16
ED7
ED16
ED16
chDab1
actin
B
Retina
ED4
ED7
ED16
116
97
66
45
31
chDab1
actin

Fig. 2.2 - Nucleotide (nt) sequences of the coding regions of early and late chDab1. The early form of *chDab1* (*ChDab1-E*) is 1608 nt long including a 57 nt insertion region (INS) at nt 618 (magenta). *ChDab1-L*(late) is 1656 nt long and includes a 105 nt region deleted (DEL) in *chDab1-E* at nt 558 (boxed callout). The 57 nt insertion region is not present in *chDab1-L*. The amino acid sequence is shown for the region spanning the tyrosine phosphorylation domains with individual tyrosine phosphorylation motifs indicated in blue and the three tyrosines deleted in chDab1-E indicated in red. The indicated deletion results in the conversion of Y^{185}QTI to Y^{185}QVP. The primers used for RT-PCR (P1-P6) and splice form-specific *in situ* hybridizations (DEL: P7, P8; INS: P9, P10) are indicated. Sequence data for *chDab1-E* and *chDab1-L* have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY242122 and AY242123, respectively.

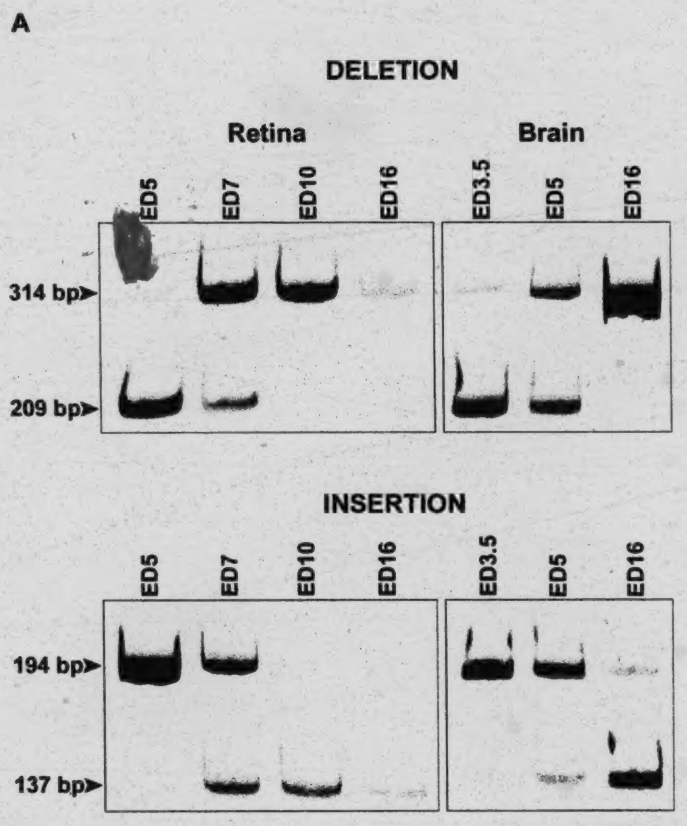
```
           P5 ➡
    ATGTCAACTGAGACAGAACTTCAAGTGGCTGTTAAAACCAGCACAAAAAAAGACTCCAAA     60
    AAGAAAGGTCAGGATCGCAGTGAAGCCACTTTAATAAAGAGGTTTAAAGGTGACGGTGTC    120
    CGATACAAAGCAAAGCTGATTGGGATCGATGAGGTTTCTGCCGCACGGGGAGACAAGTTA    180
    TGCCAGGACTCCATGATGAAGCTCAAGGGAATTGTTGCTGCGGCTCGTTCGAAAGGAGAG    240
    CATAAACAAAAAATCTTCTTAACAGTCTCCTTTGGTGGAATCAAGATCTTCGATGAAAAG    300
    ACAGGGCTACTACAGCACCACCACGCAGTTCATGAGATATCGTACATTGCAAAGGATATC    360
                                         P1 ➡
    ACAGACCACCGGGCGTTTGGATATGTGTGTGGAAAGGAGGGAAATCATCGATTTGTGGCA    420
    ATAAAAACAGCCCAGGCAGCTGAACCTGTGATTCTGGACTTGCGAGATCTGTTTCAGCTC    480
    ATCTATGAACTGAAACAAAGGGAAGAAATGGAAAAAAAGGCACAAAAGGACAAGCAGTGT    540

          ACAATTTTGGAAGAAGATGTAGAAGACCCTGTATACCAGTACATTGTGTTTGA
           T  I  L  E  E  D  V  E  D  P  V  Y  Q  Y  I  V  F  E
DEL
          GGCTGGACATGAGCCAATCCGTGAGCCTGAAACAGAAGAAAACATTTATCAG
            A  G  H  E  P  I  R  E  P  E  T  E  E  N  I  Y  Q

                                                          P3 ➡
                                             ⬅ P2
            P7 ➡                    ⬅ P8
    GAACAGGCGGTATACCAGGTTCCTACCAGCCAAAAGAAGGAAGGTGTTTATGATGTGCCA    600
     E  Q  A  V  Y  Q  V  P  T  S  Q  K  K  E  G  V  Y  D  V  P

                  P9 ➡
    AAAAGTCAACCTGTAAGTCTGGAGAATGGAAACTTATTGCTGGACATTGATGAAAATCTT    660
INS             ⬅ P10
    GGTTCAGTCACTCAGGCTGTTACCCAACTAGAGCTTTTTGGGGACATGTCCACTCCTCCC    720
                                              ⬅ P4
    GATGTAACCTCTCCCCCAACTCCTGCTACTCCAGGTGATGCCTTTATCCCATCTTCATCC    780
    CAGTCACTTCCTGCTAGTACAGACATGTTTGGTTCTGTACCTTTCAGCACTGCTGCCGTA    840
    CCCTCAGGTTATGTTGCCATGGGAGCTGTTTTGCCCTCATTCTGGGGACAGCAACCACTT    900
    GTTCAACAGCAGTTGGCCATGGGTGCTCAGCCTCCAGTTGCTCAGGTGATGCAGGGAGGA    960
    CAGCCAATAGCGTGGGGCCAACCCGGTATTTTTCCTCCTGCCCAGCAGCCGTGGCCGTCT   1020
    GTAGCTGGTCAGTTCCAACCGACTGCCTTCATGCCAACACAAACTGTTTTGCCTTTACAA   1080
    GCAGCCATGTTTCAAGGTACCATCGCTCCTATAGCTACTGTTCCACCCACAAGCGATTCC   1140
    AACAGATCAAGTCCGCAGACAGACAGGCCCAGACAGAAAATGGGAAAAGAAATGTTTAAA   1200
    GATTTCCAGATGGCTCAGCCTCCACCAGTGCCATCAAGAAAACCAGATCAGCCTTCCCTC   1260
    AGTTGTACCTCAGAGGCCTTCTCAAGTTACTTTAACAAAGTTGGGATGGCACAGGAAGCA   1320
    GATGATTGTGATGACTTTGACATCTCTCAGTTAAATTTGACTCCTGTGACTTCCACGACA   1380
    CCATCAACAAACTCACCTCCAACCCCTGCACCCAGACAGAGTTCTCCATCGAAATCATCA   1440
    GCATCTCATACCAGCGATCCTGCTGCAGATGACCTCTTTGAAGAGGGGTTTGAAAGCCCA   1500
    AGCAAAAGTGAGGAGCAGGAGGCTCCTGATGAGTCTCAGGCCTCATCCAACAGTGATCCA   1560
                                        ⬅ P6
    TTTGGTGAGCCAACTGGTGATACTATAAGTCCACAGGTCGGTAGCTAG               1608
```

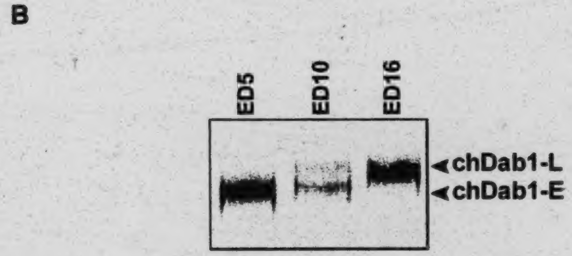
alternative splicing of the 105 bp deletion region has not been previously reported, alternative splicing of the 57 bp exon has been described in chicken, as well as in mouse where this exon is duplicated (Bar *et al.*, 2003)

To further investigate the possibility of alternative splicing in *chDab1*, RT-PCR analysis of retina and brain at different developmental stages was carried out using primer pairs flanking the deletion region (P1/P2) and the insertion region (P3/P4) (Fig. 2.2). As shown in Fig. 2.3A, RT-PCR of the deletion region generated two DNA bands, of 209 bp and 314 bp. The 209 bp band was predominant in the ED5 retina, while the 314 bp band was most abundant in the ED10 and ED16 retina, with a barely detectable 209 bp band at ED10. An intermediate banding pattern was observed in the ED7 retina. A similar pattern was seen in the developing brain, with both bands observed at ED3.5 and ED5, and the 314 bp band being predominant at ED16. Next, we studied the insertion region by RT-PCR. Two bands of 194 bp and 137 bp were observed. The higher band was predominant in ED5 retina and ED3.5 brain. By ED16, the 137 bp band was more intense in both retina and brain.

DNA isolated from the 314 bp, 209 bp, 194 bp and 137 bp bands was sequenced. As expected, the 209 bp and 194 bp bands, predominant at early stages of retina and brain development, exclude the 105 bp deletion region (DEL) and include the 57 bp insertion region (INS), respectively (Fig. 2.2). This previously uncharacterized form has been labeled *chDab1-E*. The 314 bp and 137 bp bands, predominantly found at later stages of development, include the

Fig. 2.3 - RT-PCR analysis of chDab1 deletion and insertion regions. (**A**) cDNAs synthesized from poly(A)$^+$ RNA from retina (ED5, ED7, ED10, ED16) and brain (ED3.5, ED5, ED16) were amplified using P1 and P2 primers for deletion analysis and P3 and P4 primers for insertion analysis. Sizes of amplified bands are indicated. (**B**) Banding patterns obtained by RT-PCR analysis of ED5, ED10 and ED16 retina using P1 and P4 primers. The lower band is 382 bp and the higher band is 430 bp.


A
DELETION
Retina
Brain
ED5
ED7
ED10
ED16
ED3.5
ED5
ED16
314 bp
209 bp
INSERTION
ED5
ED7
ED10
ED16
ED3.5
ED5
ED16
194 bp
137 bp


B
ED5
ED10
ED16
chDab1-L
chDab1-E

deletion region and exclude the insertion region, respectively. This form, well-documented in human and mouse, has been labeled *chDab1-L*.

The conversion from *chDab1-E* to *chDab1-L* involves two alternative splicing events. To determine whether these splicing events occur at the same time during retinal development, RT-PCR analysis was carried out using primer set P1 and P4, which spans the deletion/insertion region (Fig. 2.2). Simultaneous splicing events would result in only two amplified bands: (i) a band of 430 bp including the 105 bp deletion region and excluding the 57 bp insertion region, and (ii) a band of 382 bp excluding the deletion region and including the insertion region. If the insertion/deletion events occurred at different developmental stages, intermediate products of 487 bp or 325 bp would be observed. RT-PCR analysis of ED5, ED10 and ED16 retina generated only two bands of 382 bp and 430 bp (Fig. 2.3B). The ED10 retina had both bands, while ED5 and ED16 had the 382 bp band and the 430 bp band, respectively. All four bands were isolated and sequenced to confirm our predictions.

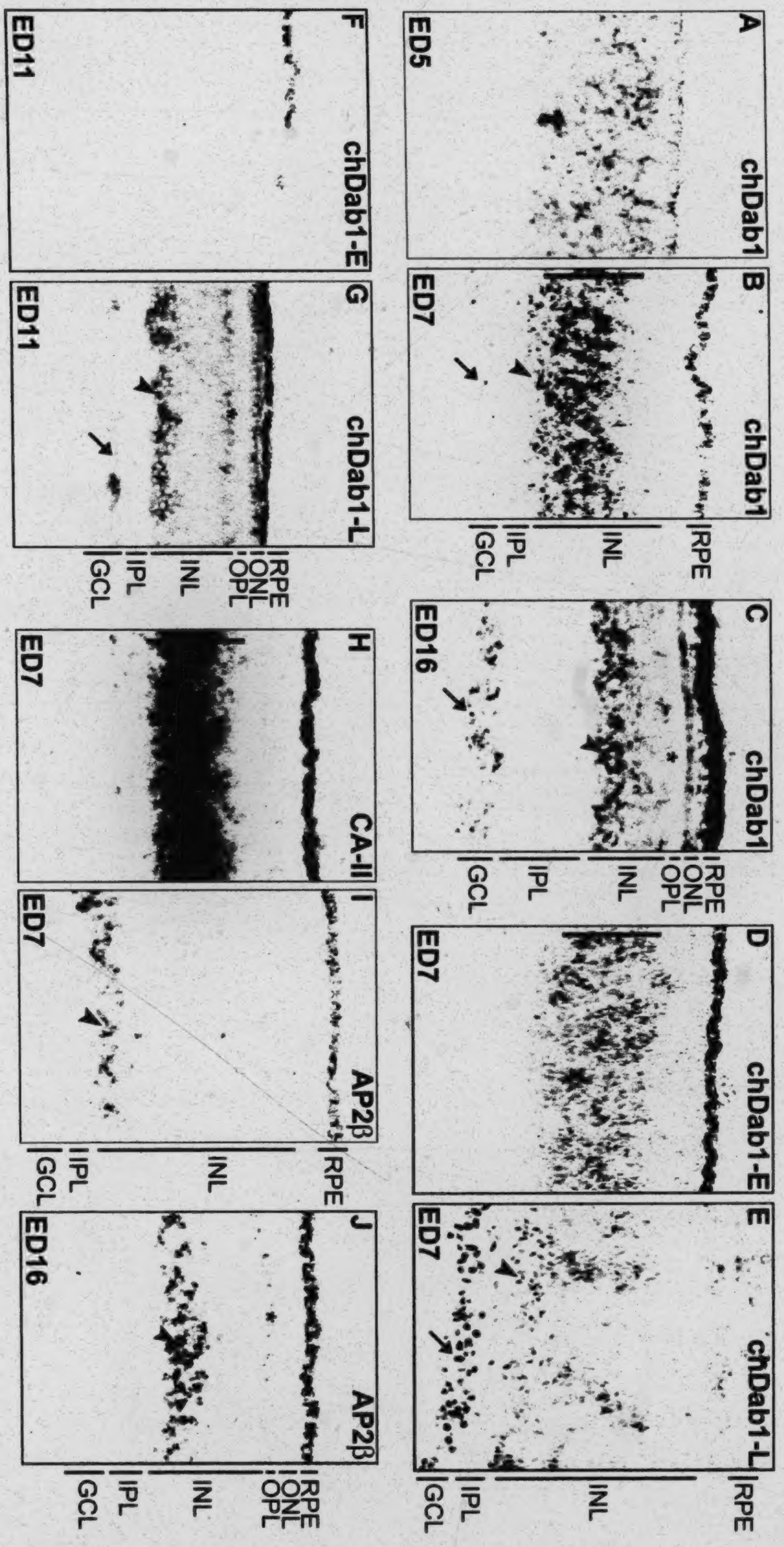
Examination of the chDab1-E deletion and insertion regions reveals some important clues as to the function of these domains. The 35 aa deletion region overlaps with three nonreceptor tyrosine kinase (NTK) recognition sites: $Y^{185}QTI$, $Y^{198}QY^{200}I$ and $Y^{220}QVP$ (Howell *et al.*, 1997a; Howell *et al.*, 2000; Keshvara *et al.*, 2001), with the $Y^{185}QTI/Y^{220}QVP$ motifs converted into YQVP as a result of the deletion (Fig. 2.2). Tyr-198 and tyr-220 have previously been shown to be the major sites for Reelin-induced Dab1 phosphorylation in embryonic neurons (Howell *et al.*, 1999; Keshvara *et al.*, 2001). The 19 aa insertion region has no homology to any known motifs; however, it does show some similarity (11/19 aa) to an exon found in Dab1-related proteins: murine p96/p67 (Xu et al., 1995), also known as Disabled-2 (Dab2), and the human DOC-2 (Albertsen et al., 1996), suggesting consolidation of some Dab2 function in chDab1-E.

2.3.3 ChDab1-E and chDab1-L RNAs are expressed in undifferentiated and differentiated retinal cells, respectively

Retinal tissue sections were examined by *in situ* hybridization to determine the distribution pattern of *chDab1* RNA. At ED5, when 85% of cells are undifferentiated and proliferating (Dutting *et al.*, 1983), *chDab1*-positive cells were found throughout the retina (Fig. 2.4A). Two days later (ED7), *chDab1*-positive cells were found in the inner two thirds of the inner nuclear layer (INL), and in the ganglion cell layer (Fig. 2.4B). At this developmental stage, proliferating cells (~50% of total) are located in the central portion of the INL. The emerging photoreceptor layer next to the retinal pigmented epithelium (RPE) was negative. In the differentiated ED16 retina, *chDab1*-positive cells were primarily found in the ganglion cell layer (GCL) and the innermost third of the INL where amacrine cells are located (Fig. 2.4C). There was also a weak signal in the outer nuclear layer (ONL) containing photoreceptor cells as well as in the outermost layer of cells in the INL where horizontal cells are located.

Using primers flanking the deletion and insertion regions, we generated splice form-specific probes to study the pattern of expression of *chDab1-E* and *chDab1-L* transcripts in the differentiating retina. The sizes of these probes were

Fig. 2.4 - Localization of *chDab1* transcripts in the developing chick retina. *In situ* hybridization was performed to identify the cell types expressing *chDab1* mRNA. Frozen sections of ED5 (**A**), ED7 (**B, D, E, H, I**), ED11 (**F, G**) and ED16 (**C, J**) retina were hybridized with DIG-labelled *chDab1* (**A-C**), splice form-specific *chDab1-E* (**D, F**), splice form-specific *chDab1-L* (**E, G**), CA-II (**H**) and *AP-2*β (**I, J**) antisense RNA. *ChDab1* sense RNA served as the negative control (data not shown). The DIG signal was detected using anti-DIG antibody and alkaline-phosphatase-coupled secondary antibody. The purple colour was generated using BCIP and NBT. Panel **C** was counterstained with ethyl green to show cell layers. All sections were photographed using a 20X objective. The arrows point to the ganglion cell layer, the arrowheads point to the amacrine cells in the INL, the asterisks indicate the horizontal cells in the INL and the vertical bars span the undifferentiated cells in the ED7 retina. Abbreviations are: RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.


A
chDab1
ED5
B
chDab1
ED7
RPE
INL
IPL
GCL
C
chDab1
ED16
RPE
ONL
OPL
INL
IPL
GCL
D
chDab1-E
ED7
E
chDab1-L
ED7
RPE
INL
IPL
GCL
F
chDab1-E
ED11
G
chDab1-L
ED11
RPE
ONL
OPL
INL
IPL
GCL
H
CA-II
ED7
I
AP2β
ED7
RPE
INL
IPL
GCL
J
AP2β
ED16
RPE
ONL
OPL
INL
IPL
GCL

77 nt (insertion region specific to *chDab1-E*) and 147 nt (deletion region specific to *chDab1-L*). At ED7, *chDab1-E* was detected in the central region of the INL where undifferentiated cells are located (Fig. 2.4D), while the *chDab1-L*-specific probe generated a weak signal in the inner part of the INL and a stronger signal in the ganglion cell layer (Fig. 2.4E). At ED11, no signal was detected with the *chDab1-E*-specific probe (Fig. 2.4F); however, the *chDab1-L*-specific probe generated a signal in the inner part of the INL and in the ganglion cell layer (Fig. 2.4G). In general agreement with the insertion region (*chDab1-E*) results, Bar *et al.* (2003) (Bar *et al.*, 2003) reported elevated expression of the murine equivalent of the insertion region in the proliferating cells of the ventricular zone in E14 brain, with decreased levels at P0.

To more conclusively identify the cells expressing *chDab1-E* and *chDab1-L* within the INL of the developing retina, we used two markers: carbonic anhydrase II (CA-II), expressed in proliferating precursor cells and Müller glial cells (Vardimon *et al.*, 1986; Witte and Godbout, 2002), and AP-2β, expressed in amacrine cells and horizontal cells (Bisgrove and Godbout, 1999). *In situ* hybridization of ED7 sections revealed *CA-II* mRNA in the central part of the INL (Fig. 4H), while *AP-2β* was detected in the innermost layers of the INL in both ED7 (Fig. 2.4I) and ED16 (Fig. 2.4J) retina, with a positive outermost layer of cells (horizontal cells) also seen at ED16. Taken together, the *in situ* hybridization and RT-PCR results indicate that *chDab1-E* is expressed in retinoblasts while *chDab1-L* is primarily found in amacrine and ganglion cells.

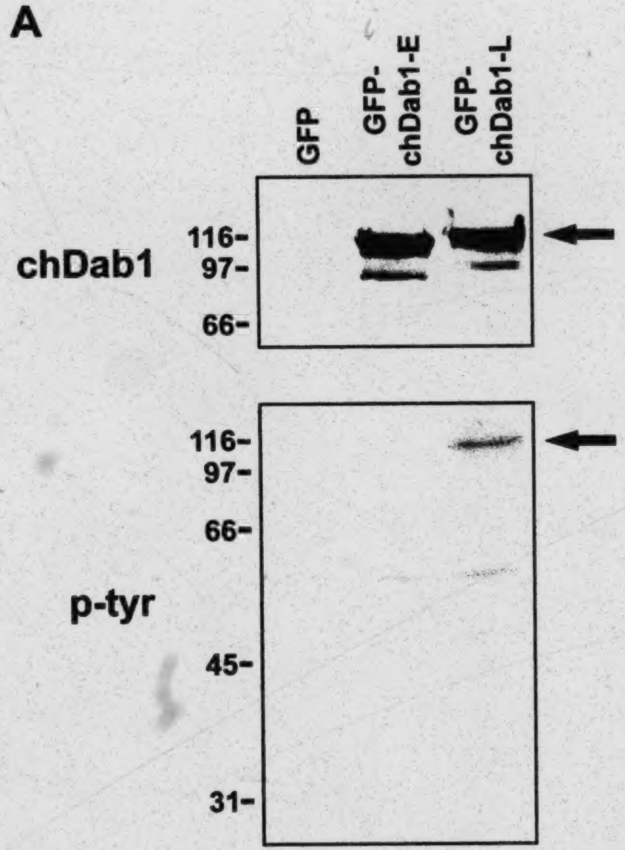
2.3.4 ChDab1-L modulates cellular morphology and tyrosine phosphorylation of retinal cells

To address the significance of having two chDab1 isoforms in the differentiating retina, the coding regions of chDab1-E and chDab1-L were generated by RT-PCR using poly(A)$^+$ RNA from ED5 and ED16 chick retina, respectively, and cloned in-frame with GFP into the pEGFP-C1 expression vector. After verification of the cDNA sequences, both expression constructs, as well as empty vector, were transfected into primary retinal cultures prepared from ED5 embryos. Western blot analysis confirmed expression of the GFP-chDab1 fusion proteins in transfected cells (Fig. 2.5A).

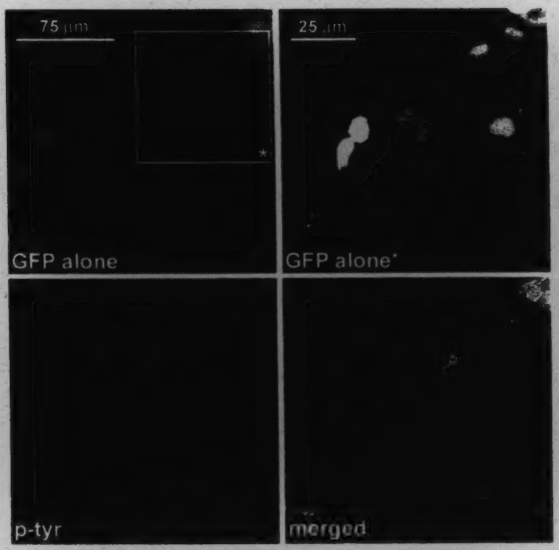
The subcellular localization of the GFP-fusion proteins was studied by confocal microscopy. Both isoforms of chDab1 had a cytoplasmic pattern (Figs. 2.5C, 2.5D). In contrast, GFP protein was found throughout the cell (Fig. 2.5B). Transfection of the chDab1-L construct was accompanied by striking morphological differences, with numerous thin elongated processes extending from most GFP-chDab1-L-positive cells (Fig. 2.5D). In addition, many of the transfected cells appeared smaller and more stringy. Cells transfected with GFP (Fig. 2.5B) and GFP-chDab1-E (Fig. 2.5C) were generally more spherical and neuroblastic, with fewer and less elongated processes.

Since a major difference between the late and early forms of chDab1 is the lack of tyrosine phosphorylation sites predicted to be necessary for Dab1 activation and relaying of the Reelin-Dab1 signal, we next looked at tyrosine phosphorylation in the transfected cells. As shown in Fig. 2.5D, a strong

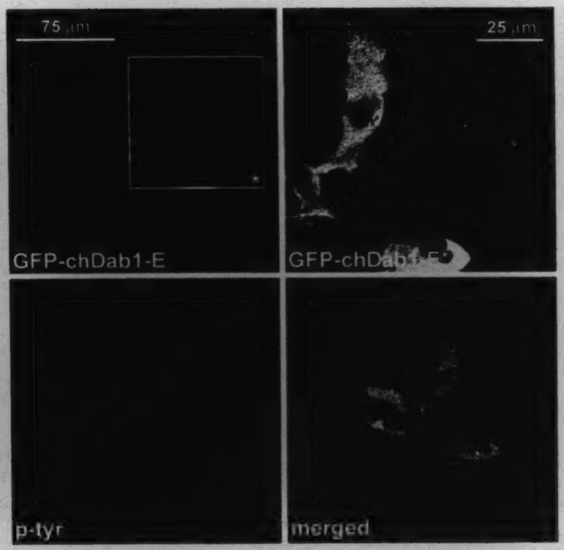
Fig. 2.5 - Analysis of chDab1-transfected primary retinal cultures. (**A**) Western blot analysis of GFP, GFP-chDab1-E and GFP-chDab1-L transfected retinal cells. Cell extracts (30 μg/lane) were electrophoresed in a 10% SDS-PAGE gel and blotted onto a nitrocellulose filter. GFP-chDab1 expression was detected using anti-Dab1 antibody (1:5000) while phosphotyrosine was detected using anti-phosphotyrosine antibody (1:1000). GFP-chDab1 and tyrosine phosphorylated GFP-chDab1 proteins are indicated by an arrow. Molecular mass standards (in kDa) are shown on the left. (**B-I**) GFP expression and phosphotyrosine (p-tyr) in GFP-chDab1 transfected retinal cultures. Cells were transfected with expression constructs encoding GFP (**B**), GFP-chDab1-E (**C**), GFP-chDab1-L (**D**), GFP-chDab1-L^{Y185F} (**E**), GFP-chDab1-L^{Y198F} (**F**), GFP-chDab1-L^{Y200F} (**G**), GFP-chDab1-L^{Y220F} (**H**) and GFP-chDab1-L$^{Y185F/Y198F}$ (**I**). Cells were fixed with 4% paraformaldehyde, permeabilized and stained with anti-phosphotyrosine antibody and Alexa 555 goat anti-mouse secondary antibody. The GFP signal in transfected cells was detected by epifluorescence. Boxed regions (*) are enlargements to show morphological details of transfected cells.


A
GFP
GFP-chDab1-E
GFP-chDab1-L
chDab1
116-
97-
66-
p-tyr
116-
97-
66-
45-
31-

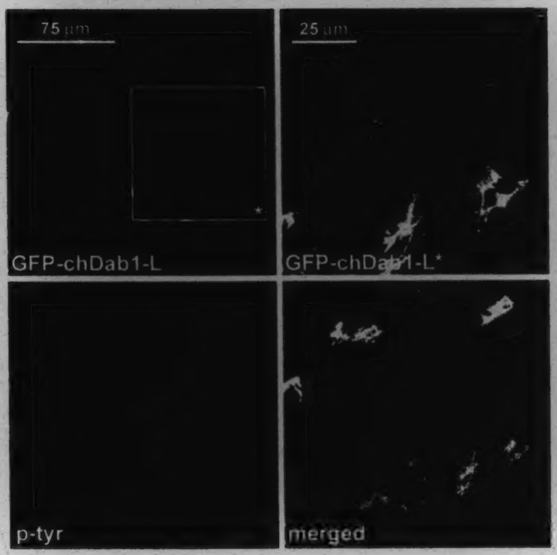
B


75 μm
25 μm
GFP alone
GFP alone*
p-tyr
merged

C


75 μm
25 μm
GFP-chDab1-E
GFP-chDab1-E*
p-tyr
merged

D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25 μm
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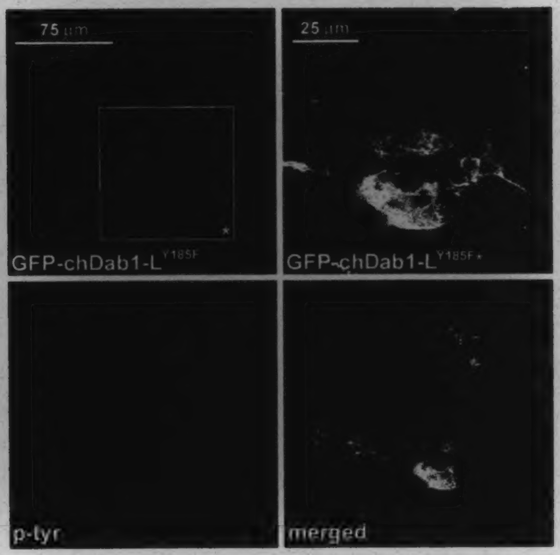
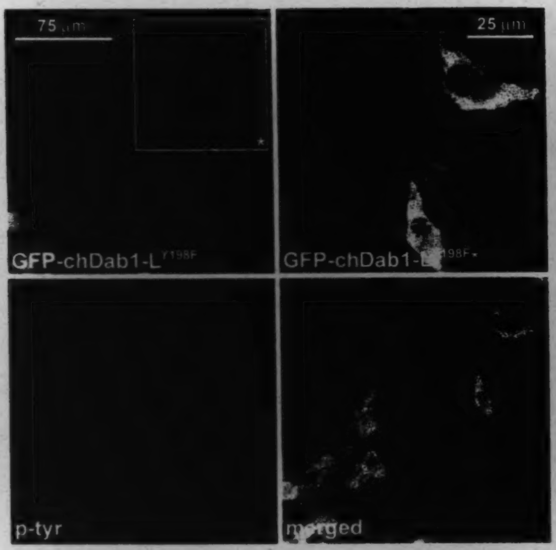
GFP-chDab1-L*
p-tyr
merged

E


75 μm
25 μm
GFP-chDab1-L^{Y185F}
GFP-chDab1-L^{Y185F*}
p-tyr
merged

F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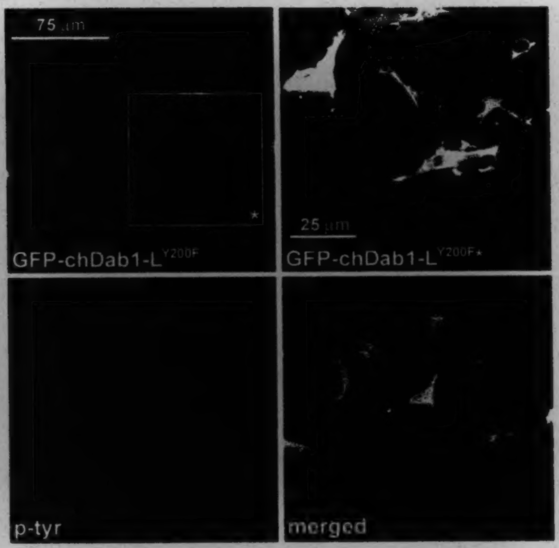
GFP-chDab1-L^{Y198F}
25 µm
GFP-chDab1-L^{Y198F}*
p-tyr
merged

G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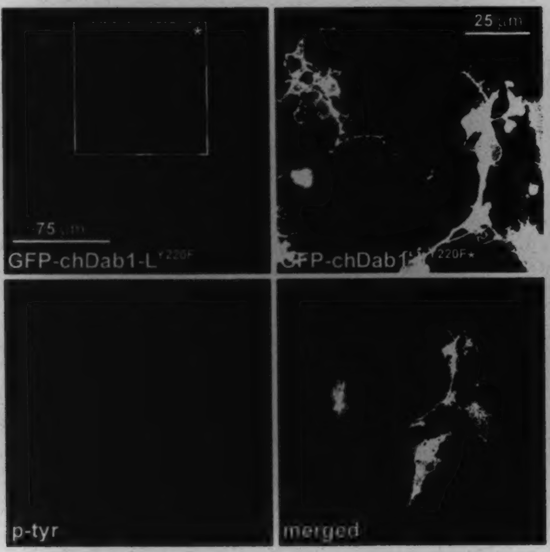
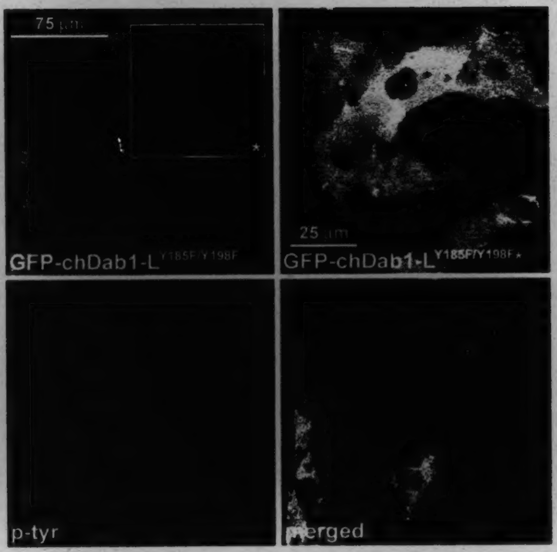
GFP-chDab1-L^{Y200F}
25 µm
GFP-chDab1-L^{Y200F}*
p-tyr
merged

H


75 µm
GFP-chDab1-L^{Y220F}
25 µm
GFP-chDab1-L^{Y220F}*
p-tyr
merged

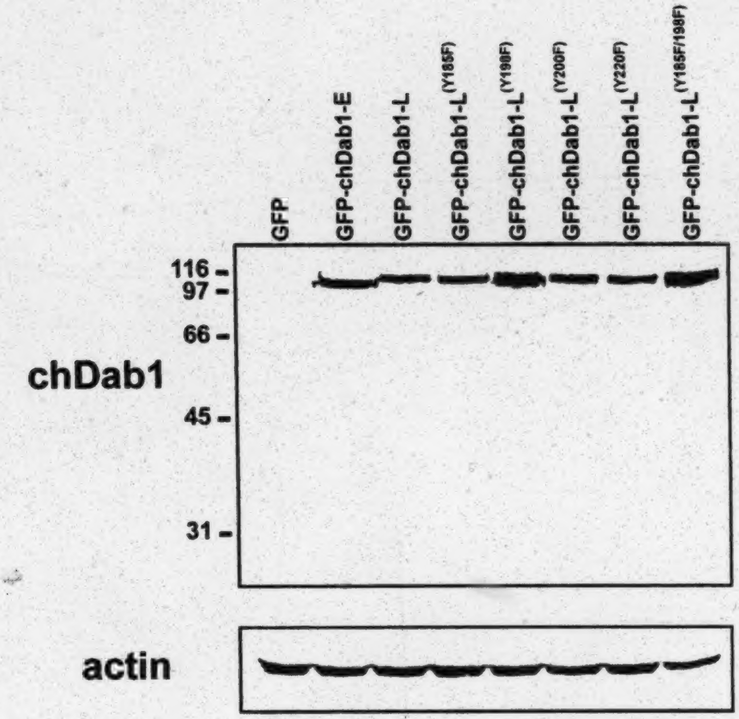
I


75 µm
GFP-chDab1-L$^{Y185F/Y198F}$
25 µm
GFP-chDab1-L$^{Y185F/Y198F}$*
p-tyr
merged

phosphotyrosine signal was observed in GFP-chDab1-L-positive retinal cells. In contrast, no phosphotyrosine signal above background was detected in either GFP-chDab1-E transfected cells (Fig. 2.5C) or GFP control (Fig. 2.5B). Phosphorylation of chDab1-L was confirmed by western blotting of cellular extracts derived from transfected cells with anti-phosphotyrosine antibody (Fig. 2.5A). These results suggest a specific role for the chDab1-L isoform in relaying tyrosine phosphorylation signals which affect cellular morphology.

To study the role of the four tyrosine residues located within or in the immediate vicinity of the two exons deleted in chDab1-E, we mutated each of these residues in the GFP-chDab1-L expression construct by converting the tyrosine into a phenylalanine. Retinal cells transfected with wild-type and mutant constructs were analyzed by western blotting to ensure that full-length GFP-fusion proteins accumulated to the same levels in transfected cells (Fig. 2.6), as well as by confocal microscopy. As shown in Fig. 2.5E, mutation of tyr-185 produced cells with a similar appearance to those transfected with wild-type chDab1-L. Mutation of tyr-198 abolished tyrosine phosphorylation and produced cells similar in appearance to chDab1-E-transfected cells (Fig. 2.5F). Mutation of tyr-200 generated an intermediate phenotype, with some tyrosine phosphorylation detected above background and a moderate number of processes in transfected cells (Fig. 2.5G). The fourth mutation, tyr-220, had no effect on either the morphology or phosphorylation status of transfected cells (Fig. 2.5H). As expected, cells transfected with an expression construct mutated

Fig. 2.6 - Western blot analysis of wild-type and mutant GFP-chDab1 transfected retinal cells. Extracts (50 mg/lane) from cells transfected with each of the indicated constructs were electrophoresed in a 10% SDS-PAGE gel and blotted onto a nitrocellulose filter. GFP-chDab1 expression was detected using anti-Dab1 antibody (1:5000). Immunoblotting with anti-actin antibody (1:500) served as a control for equal protein loading. Molecular mass standards (in kDa) are shown on the left.


GFP
GFP-chDab1-E
GFP-chDab1-L
GFP-chDab1-L(Y185F)
GFP-chDab1-L(Y198F)
GFP-chDab1-L(Y200F)
GFP-chDab1-L(Y220F)
GFP-chDab1-L(Y185F/198F)
116
97
66
45
31
chDab1
actin

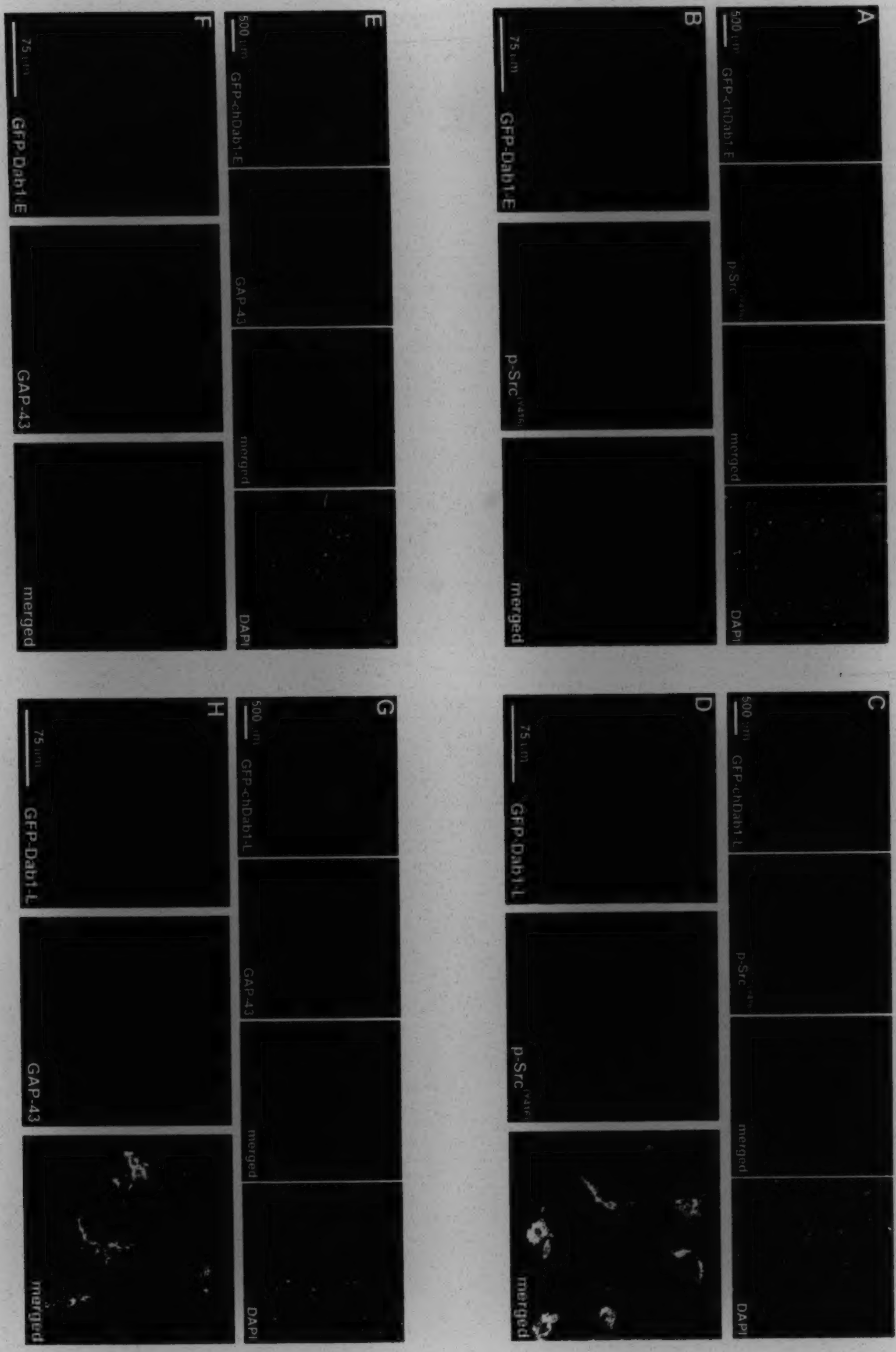
at both tyr-185 and tyr-198 were similar in appearance to those transfected with chDab1-E (Fig. 2.5I).

2.3.5 ChDab1-L expression results in Src activation and induction of GAP-43

Dab1 tyrosine phosphorylation activates Src family kinases. To address whether expression of chDab1-L results in Src family kinase activation, GFP-chDab1-E and GFP-chDab1-L transfectants were stained with an antibody to phosphorylated Src (tyr-416) predicted to recognize all Src family kinases phosphorylated at this residue. GFP-chDab1-L transfected cells showed strong staining with this antibody compared to GFP-chDab1-E transfected cells (Figs. 2.7A-D) suggesting that at least some of the proteins detected with the antibody to phosphotyrosine represent activated Src family kinases. Analysis of chDab1-L mutants generated the expected results; i.e., activation of Src family kinases in cells transfected with chDab1-L$^{(Y185F)}$, chDab1-L$^{(Y220F)}$, and to a lesser extent chDab1-L$^{(Y200F)}$. However, no induction of phospho-Src$^{(416)}$ was observed in cells transfected with the chDab1-L$^{(Y198F)}$ mutant (data not shown).

Neuronal maturation is accompanied by neurite outgrowth. To determine whether the enhanced formation of processes observed in chDab1-L transfectants was associated with a more differentiated state, transfected cells were stained with an antibody to neurofilament-H, the heavy subunit of the major intermediate filaments found in the axons of mature neurons. Neurofilament-H was expressed in a significant proportion of both transfected and non-transfected

Fig. 2.7 - Phospho-Src and GAP-43 analysis of chDab1-transfected primary retinal cultures. GFP-chDab1-E (**A, B, E, F**) and GFP-chDab1-L (**C, D, G, H**) transfected retinal cultures were immunostained with anti-phospho-Src$^{(416)}$ (**A-D**) or anti-GAP-43 (**E-H**) antibodies followed by Alexa 555 goat anti-mouse secondary antibody. (**A, C, E, G**) show a low magnification view of the transfected cultures taken with a 2.5X objective to demonstrate the overall extent of p-Src$^{(416)}$ and GAP-43 staining. (**B, D, F, H**) show a high magnification view of individual clumps of cells taken with a 40X objective.


A
500 µm
GFP-chDab1-E
p-Src Y416
merged
DAPI
B
75 µm
GFP-Dab1-E
p-Src Y416
merged
C
500 µm
GFP-chDab1-L
p-Src Y416
merged
DAPI
D
75 µm
GFP-Dab1-L
p-Src Y416
merged
E
500 µm
GFP-chDab1-E
GAP-43
merged
DAPI
F
75 µm
GFP-Dab1-E
GAP-43
merged
G
500 µm
GFP-chDab1-L
GAP-43
merged
DAPI
H
75 µm
GFP-Dab1-L
GAP-43
merged

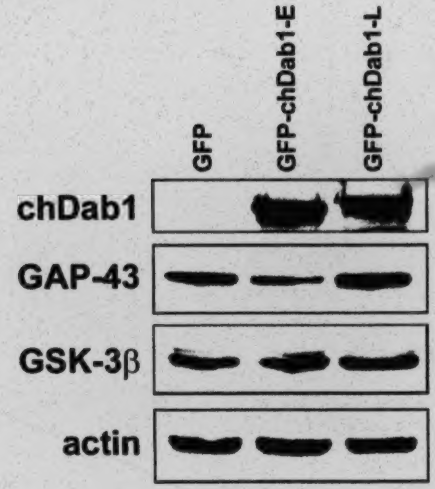
retinal cells, with no obvious differences in staining patterns observed between chDab1-E and chDab1-L transfectants (data not shown).

Growth-associated protein (GAP)-43 is a neuronal phosphoprotein associated with axonal outgrowth during development, regeneration and sprouting. In the retina, GAP-43 is primarily found in the axons of ganglion cells, but has also been reported in the processes of amacrine cells (Kapfhammer et al., 1997). A difference in staining pattern was observed between chDab1-E and chDab1-L transfectants using anti-GAP-43 antibody, with a considerably stronger signal observed in GFP-chDab1-L-expressing cells (Figs. 2.7E-H). These results are in agreement with western blot analysis of whole cell extracts isolated from GFP-chDab1 transfected cells (Fig. 2.8). The intensity of the GAP-43 band was ~2-fold stronger in chDab1-L transfectants than in chDab1-E and GFP (control) transfectants. In contrast, signal intensity in all three transfectants was virtually identical for GSK-3β and actin.

2.3.6 ChDab1-L-mediated Src activation and process formation require Reelin signaling

Activation of SFKs and neuronal migration require Reelin binding to its receptors thereby inducing Dab1 tyrosine phosphorylation. To determine whether SFK activation and the formation of neurite-like processes in chDab1-L-expressing cells is Reelin-dependent, we treated GFP-Dab1-L transfected cells with the Reelin inhibiting antibody, CR-50. CR-50 has previously been shown to interfere with Reelin-receptor binding thereby preventing Reelin-mediated

Fig. 2.8 - Induction of GAP-43 in GFP-chDab1-L-transfected retinal cells. Extracts (50 mg/lane) from cells transfected with each of the indicated constructs were electrophoresed in a 10% SDS-PAGE gel and blotted onto a nitrocellulose filter. The filter was sequentially immunoblotted with anti-Dab1 antibody (1:5000), anti-GAP-43 antibody (1:500) and anti-GSK-3β antibody (1:2500). Immunoblotting with anti-actin antibody (1:500) served as a control for equal protein loading.


GFP
GFP-chDab1-E
GFP-chDab1-L
chDab1
GAP-43
GSK-3β
actin

signaling (Ogawa *et al.*, 1995; D'Arcangelo *et al.*, 1997). As expected, mock-treated Dab1-L-expressing cells (Fig. 2.9A) displayed robust phospho-Src^{416} immunoreactivity and formed processes while chDab1-L-expressing cells treated with CR-50 for two days failed to form processes and showed reduced immunoreactivity to anti-phospho-Src$^{(416)}$ (Fig. 2.9B). The appearance of CR-50-treated chDab1-L-expressing cells was similar to that of chDab1-E-expressing cells. The absence of SFK activation and the suppression of Dab1-L-induced morphology by CR-50 indicate that Reelin signaling is required for Dab1-L function in the retina.

2.3.7 ChDab1 isoform switching corresponds to increased levels of phosphotyrosine, activated Src and GAP-43

To determine whether expression of chDab1-L correlates *in vivo* with presence of phosphotyrosine, activated Src family kinases and GAP-43, ED5 and ED16 retinal tissue sections were immunostained with antibodies to Dab1, phosphotyrosine, phospho-Src$^{(Y416)}$ and GAP-43. At ED5 (when chDab1-E is predominant), a strong Dab1 cytoplasmic signal was observed throughout the retina (Figs. 2.10A-C). Phosphotyrosine (Fig. 2.10A), phospho-Src (Fig. 2.10B) and GAP-43 (Fig. 2.10C) were mainly found in the emerging NFL containing the axons of ganglion cells. At ED16 (when chDab1-L is predominant), Dab1 was primarily expressed in the NFL and IPL, with substantial expression in ganglion cells and in the inner half of the INL where the elongated stringy staining pattern was consistent with expression in the cytoplasm and processes of amacrine cells

Fig. 2.9 - Analysis of mock- (**A**) and CR-50-treated (**B**) Dab1-L-expressing retinal cells. GFP-Dab1-L-transfected retinal cells were treated with CR-50 antibody and immunostained with anti-phospho-Src^{416} (p-Src^{416}) antibody followed by Alexa 555-conjugated goat anti-mouse secondary antibody. The GFP signal in transfected cells was detected by epifluorescence.

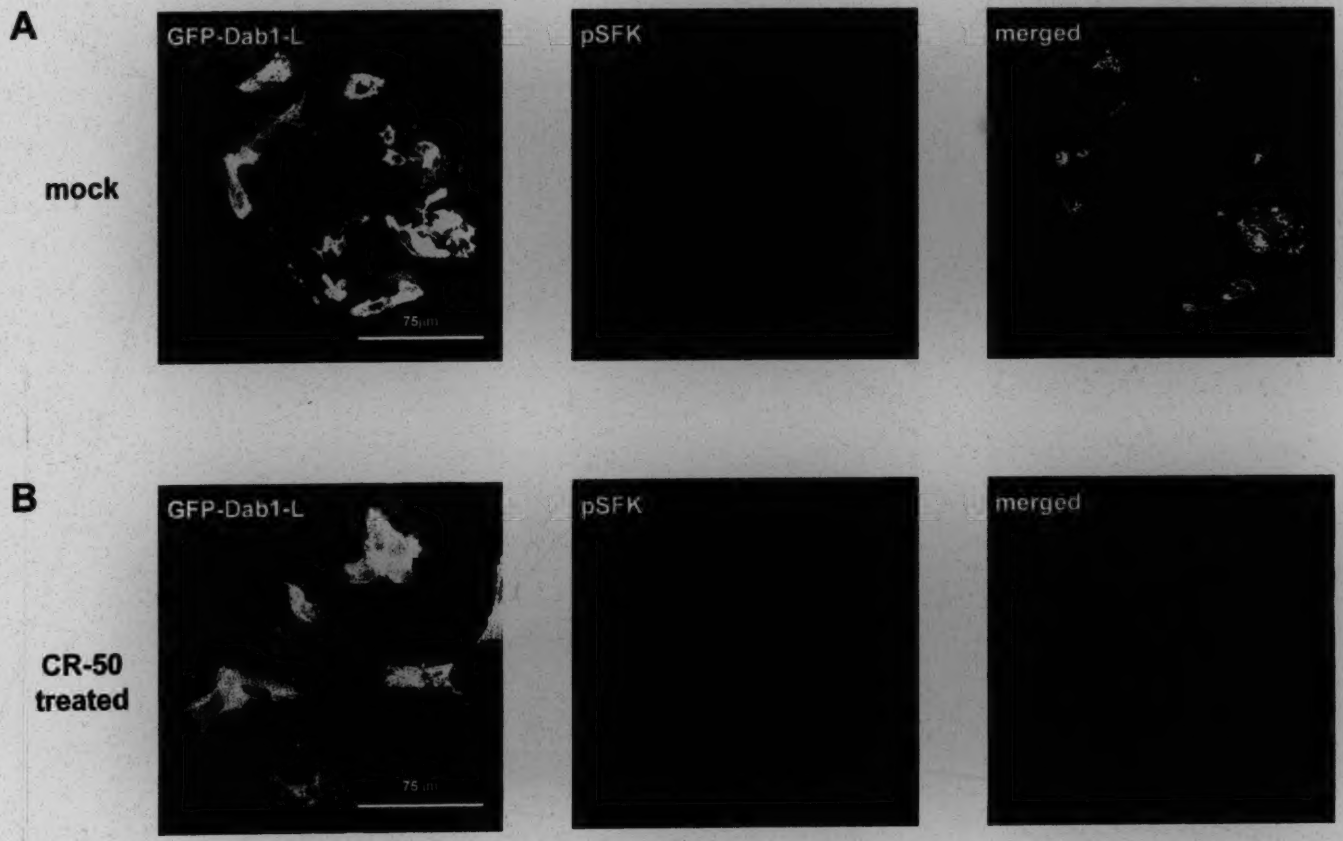
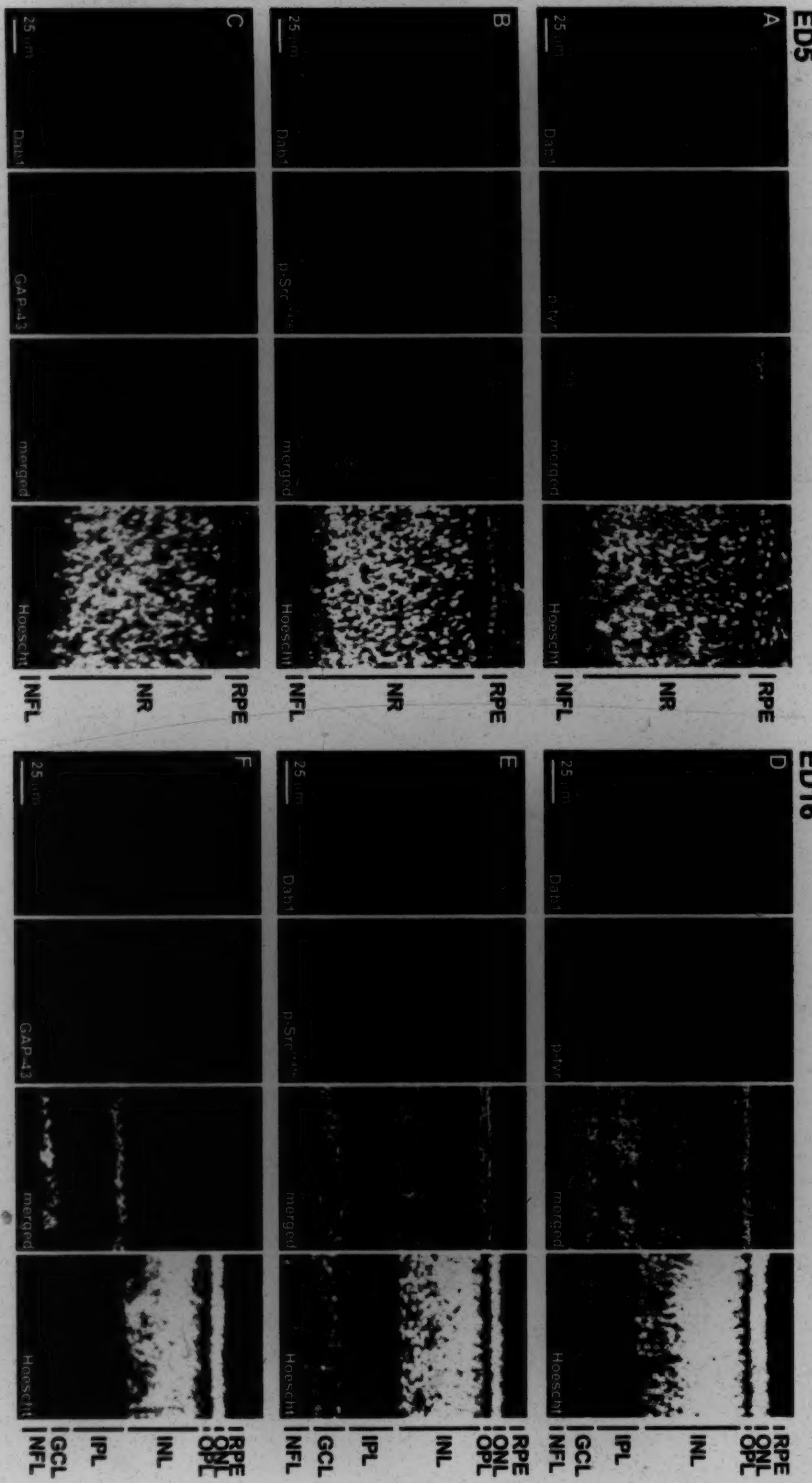

A
mock
GFP-Dab1-L
75µm
pSFK
merged
B
CR-50
treated
GFP-Dab1-L
75 µm
pSFK
merged

Fig. 2.10 - Immunofluorescence analysis of chDab1, phosphotyrosine, phosphorylated Src family kinases and GAP-43 in the developing chick retina. Sections were double-stained with anti-Dab1 and anti-phosphotyrosine, anti-phospho-Src$^{(416)}$ [p-Src$^{(416)}$)] or anti-GAP-43 antibodies, followed by counterstaining with the fluorescent dye Hoescht 33258 to label the nuclei. Retinal tissue sections were prepared from ED5 (**A, B, C**) and ED16 (**D, E, F**) embryos. Abbreviations are: RPE, retinal pigment epithelium; NR, neural retina; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.


ED5
ED16
A
B
C
D
E
F
25 µm
Dab1
ptyr
p-Src
GAP-43
merged
Hoescht
RPE
NR
NFL
ONL
OPL
INL
IPL
GCL

(Figs. 2.10D-F). Phosphotyrosine had a similar distribution pattern, although the signal was comparatively stronger in the OPL (Fig. 2.10D). Phospho-Src expression was strong in ganglion and amacrine cells, although an equally strong signal was also detected in the INL cells (presumably horizontal) adjacent to the OPL (Fig. 2.10E). GAP-43 was predominant in the NFL and in the IPL immediately adjacent to the amacrine cells (Fig. 2.10F). These results indicate a strong correlation between Dab1, phosphotyrosine, activated Src and GAP-43 expression at ED16, with all four expressed in ganglion and amacrine cells and/or their processes.

2.4 DISCUSSION

We report the isolation of two alternatively spliced forms of *Dab1* from the developing chick retina: an early form expressed in the undifferentiated precursor cells of the retina and a late form found in amacrine and ganglion cells. Although more abundant in retina, these two forms are also observed in the developing brain. ChDab1-L encodes a predicted protein of 551 aa and is highly similar throughout its length to human and murine Dab1. In contrast, chDab1-E encodes a predicted protein of 535 aa that contains a deletion of 35 aa and an insertion of 19 aa. While the 19 aa insertion has no recognizable features, the 35 aa deletion results in the loss of tyrosine phosphorylation sites implicated in Reelin-Dab1-mediated signal transduction (Howell *et al.*, 1997a; Keshvara *et al.*, 2001; Arnaud *et al.*, 2003; Bock and Herz, 2003). To date, mammalian counterparts of chDab1-E containing both the 35 aa deletion and the 19 aa insertion have not been described; however, Bar *et al.* (2003) have shown by RT-PCR analysis that exons encoding the 19 aa insertion are transcribed in ED11/12 mouse brain as well as in ED6 chick brain. These exons were not transcribed in later stage embryos or in primary cortical neuron cultures.

Reelin and Dab1 have been shown to play a central role in the positioning of migrating neurons in the cerebral cortex, cerebellum and hippocampus (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Howell *et al.*, 1997b). Like the brain, the retina is a highly organized laminated structure characterized by migration of neuronal cells during development, their positioning into specific layers and communication from one nuclear layer to the next through specific synaptic circuitry. Detailed structural analysis of the retina in Reelin-deficient mice has revealed a number of abnormalities, including a decrease in the density of rod bipolar cells, a reduced density of dendrites in the IPL which could not be attributed to reduced amacrine cell density, and alteration in the layering of amacrine cell processes in the IPL (Rice and Curran, 2000; Rice *et al.*, 2001).

In support of a role for the Reelin-Dab1 signaling pathway in the retina, Reelin expression is elevated in the ganglion cells of the mouse, chicken, turtle and lizard retina (Schiffmann *et al.*, 1997; Bernier *et al.*, 1999; Goffinet *et al.*, 1999; Bernier *et al.*, 2000). Dab1 has been detected in the mouse retina shortly after birth and is primarily found in a single layer of amacrine cells, called glycinergic type AII, located immediately next to the IPL (Rice and Curran, 2000). The IPL, containing the processes of Dab1-positive amacrine cells, is also strongly positive for Dab1, with two distinct zones corresponding to the On and

Off sublayers. Similar to the mouse retina, we have found chDab1-L expression in amacrine cells, with particularly high levels in the IPL. However, there are a number of differences between mouse and chicken: (i) chick retinal precursor cells express elevated levels of chDab1-E, (ii) chDab1-L is expressed in ganglion cells, and (iii) rather than a single layer of amacrine cells, chDab1-L extends through several layers of amacrine cells. Our results suggest a more widespread role for Dab1 in the chick retina than in mouse retina, involving retinoblasts and ganglion cells as well as amacrine cells.

To address the role of *chDab1* alternative splicing in chick retina development, we overexpressed chDab1-E and chDab1-L in primary retinal cultures. Our results showed strong induction of tyrosine phosphorylation and Src phosphorylation, specific to chDab1-L-transfected cells. These phosphorylation events were associated with the formation of numerous thin elongated cellular processes reminiscent of neurite outgrowth, a hallmark of differentiated neuronal cells. Furthermore, Src activation and neurite outgrowth were found to be dependent on Reelin signaling as CR-50-mediated inhibition of Reelin in chDab1-L-expressing cells produced a chDab1-E-like phenotype. No significant differences in neurofilament-H staining patterns were observed between chDab1-E and chDab1-L transfectants; however, levels of the axonal protein GAP-43 were significantly increased in chDab1-L transfectants. GAP-43 expression correlates with neurite outgrowth and is postulated to play a fundamental role in elongation and/or guidance of axons by regulating the response of growing axons to intra- and extra-cellular signals (Benowitz and Routtenberg, 1997).

Overexpression and depletion of GAP-43 have previously been associated with formation of neurite-like filopodia, and reduction in the size of neurites and growth cones, respectively (Aigner and Caroni, 1993; Aigner and Caroni, 1995). Our transfection data support a role for GAP-43 in neurite formation and suggest a link between the Reelin-Dab1 signaling pathway and GAP-43, mediated through activated Src family kinases. A relationship between GAP-43 and the Reelin-Dab1 pathway is supported by the fact that chDab1-L, GAP-43 and phospho-Src are found in the same differentiated cell types in retinal tissue (ganglion and amacrine and/or their processes). A role for Reelin-Dab1 in the formation of processes is in general agreement with the reduced density of dendrites observed in Reelin-deficient mice (Rice *et al.*, 2001) and with a recent report by Niu *et al.* (2004) (Niu et al., 2004) indicating that Reelin and Dab1 affect dendritic outgrowth from normal hippocampal neurons.

The expression patterns of chDab1-E and chDab1-L in the developing retina and brain, and the specific effects of chDab1-L in primary retinal cultures suggest that both forms of Dab1 may be important in retinal/brain development and differentiation. Although there are no recognizable motifs within the 19 aa insertion in chDab1-E, this insertion may alter some aspect of Dab1 function in precursor cells. The 35 aa deletion specific to chDab1-E spans a region containing three tyrosine phosphorylation sites. One of these sites, tyr-198, and the adjacent tyr-220 have been shown to undergo Reelin-induced tyrosine phosphorylation (Keshvara *et al.*, 2001). In support of a critical role for tyrosine phosphorylation in Reelin-mediated Dab1 activity, mice mutated at all five tyrosines in the tyrosine phosphorylation motifs, including tyr-198 and tyr-220, display identical phenotypes to those of *reeler* and *yotari* (Howell *et al.*, 2000). Furthermore, mouse Dab1 protein is phosphorylated specifically in the developing brain as opposed to the adult brain, at a time when cells are expanding and axonal networks are developing (Howell *et al.*, 1997a).

By mutational analysis, we have shown that tyr-198 is required for the formation of elongated processes in our transfected cells. Mutation of tyr-220 and tyr-185 residues had little or no effect on the retinal cells. The partial effect observed upon mutation of tyr-200 suggests that this residue is required for optimal recognition of the tyr-198 phosphorylation motif. In agreement with this, Keshvara *et al.* (2001) observed reduced reactivity of their anti-phospho-tyr198/tyr-200 Dab1 antibody when tyr-200 was mutated. Tyr-185 and tyr-198/tyr-200 are part of two YQXI motifs which bind to Src-like SH2 domains. Tyr-220, along with tyr-232 which resides outside the deletion region, are part of two YXVP motifs which bind to Abl/Nck/Crk-like SH2 domains (Songyang *et al.*, 1993; Howell *et al.*, 1997a). In chDab1-E, Y^{185}-Q becomes linked to V-P, thereby converting this motif from a Src-like SH2 binding domain to a Abl/Nck/Crl SH2 binding domain. Thus, in chDab1-E, the two YQXI motifs are lost, while two YXVP motifs are retained. The alternative splicing event underlying deletion of this region therefore appears to specifically target Src family kinases. We propose that loss of YQXI phoshorylation sites in chDab1-E represents a novel way of uncoupling the Reelin-Dab1 pathway, to ensure that this signaling cascade is not prematurely induced in undifferentiated retinal and brain cells by secreted Reelin. This uncoupling may be especially important in brain and retina as there is a mixture of proliferating and differentiating cells until relatively late in development. Of note, Reelin has been found to be expressed in the ganglion cells of chick retina as well as in many parts of the brain by ED6, the earliest stage tested (Bernier *et al.*, 2000). An estimated 75% of retinal cells are still in the proliferative stage at ED6 (Dutting *et al.*, 1983).

In summary, we have identified two developmentally-regulated alternatively spliced forms of chicken Dab1. We propose that Reelin-responsive chDab1-L plays a role in the formation of neurite extensions mediated through Src family kinase activation and GAP-43 expression, while chDab1-E uncouples transduction of the Reelin signal. ChDab1 isoform switching ensures specific and appropriate responses of different cell types to secreted Reelin during development. The discovery and characterization of the two chDab1 isoforms reveals a novel mechanism for regulating the Reelin-Dab1 signaling pathway and links function of a particular splice variant to a specific developmental stage.

2.5 ACKNOWLEDGEMENTS

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Chapter 3

Disabled-1 is alternatively spliced in human retina and tumours of neuroectodermal lineage

Running title: Dab1 splicing in human retina and tumours

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3.1 INTRODUCTION

Disabled-1 (Dab1) is a cytoplasmic adaptor protein that is phosphorylated when the secreted extracellular matrix glycoprotein Reelin binds to its cell surface receptors, ApoE receptor-2 (ApoER2) and very low density lipoprotein receptor (VLDLR) (D'Arcangelo *et al.*, 1997; Howell *et al.*, 1997; D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Howell *et al.*, 1999). Binding of Reelin to its receptors and the ensuing Dab1 phosphorylation stimulates Src family kinases (SFK) and the Akt serine/threonine kinase, which in turn enhances Dab1 phosphorylation (Beffert *et al.*, 2002; Arnaud *et al.*, 2003; Ballif *et al.*, 2003; Bock and Herz, 2003). Prominent roles for the Reelin-Dab1 signaling pathway include proper positioning of migrating neurons and dendrite formation in the central nervous system (CNS) (Tissir and Goffinet, 2003). Inactivation in mice of either Reelin, Dab1, or a combination of the two Reelin receptors, ApoER2 and VLDLR, results in inversion of neuronal layers in the cerebral cortex, laminar defects in the cerebellum and hippocampus, as well as altered dendrite formation (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Ware *et al.*, 1997; Trommsdorff *et al.*, 1999).

Like the brain, the retina is a highly organized laminated structure characterized by migration of neuronal cells, positioning of neuronal cells into specific layers, outgrowth of dendrites and axons, and intercellular communication through synaptic circuitry. There are six classes of neuronal cells in the retina (ganglion, amacrine, bipolar, horizontal, interplexiform and photoreceptor), located in the three nuclear layers (ganglion, inner and outer) that are separated by the inner and outer plexiform layers. Structural analysis of the retina in Reelin and Dab1-deficient mice reveals a number of abnormalities including reduced amacrine dendritic density, and alteration in the layering of amacrine cell processes in the inner plexiform layer (Rice and Curran, 2000; Rice *et al.*, 2001).

By comparing chick retinas at early and late stages of development, we have discovered a novel developmentally-regulated alternatively-spliced form of Dab1 called Dab1-E (early) that is specifically expressed in retinal progenitor cells (Katyal and Godbout, 2004). In contrast to Dab1-E, the well-characterized late form of Dab1 (Dab1-L) is expressed in two neuronal cell types: amacrine and ganglion. A key difference between the early and late forms of Dab1 is the exclusion in Dab1-E of two exons containing two Src family kinase tyrosine phosphorylation sites (Y^{185}QTI, $Y^{198}QY^{200}$I) implicated in Reelin-Dab1 signaling (Keshvara *et al.*, 2001). Of note, splicing out of the two exons results in the formation/retention of two Abl/Crk recognition sites in Dab1-E (Y^{185}QVP, Y^{232}DVP) (Songyang *et al.*, 1993; Howell *et al.*, 1997; Katyal and Godbout, 2004). Dab1-E also contains one exon that is spliced out in Dab1-L. There are no known motifs within this insertion region. Transfection of a GFP-tagged Dab1-L (late) expression construct into primary chick retinal cultures results in the formation of numerous thin elongated processes reminiscent of neurite outgrowth, increased levels of phosphotyrosine and Src family kinase activation. None of these changes are observed upon transfection of either GFP-tagged Dab1-E or GFP control expression constructs. Mutation analysis of the tyrosine phosphorylation sites in Dab1-L indicates that Src family kinase phosphorylation site Y198 is of critical importance for the morphological and biochemical alterations observed in GFP-Dab1-L-transfected cells, in agreement with previous results in mice (Howell *et al.*, 1999; Howell *et al.*, 2000; Keshvara *et al.*, 2001).

The discovery of Dab1-E has widespread implications to our understanding of the Reelin-Dab1 pathway, as the expression of Dab1-E in undifferentiated neuroblasts could serve as an effective and versatile means of ensuring that components of Reelin-Dab1 signaling are not prematurely activated in neuronal progenitor cells during retina and brain development. Here, we examine wether Dab1-E and Dab1-L exist in species other than chicken. We report that Dab1-E and Dab1-L are expressed in human fetal retina and that transfection of primary chick retinal cultures with human Dab1-L generates the same biochemical and morphological alterations observed with chicken Dab1-L. We also study Dab1 and Reelin expression in a panel of retinoblastoma (RB) and neuroblastoma (NB) cell lines and tumours to assess whether the Reelin-Dab1 pathway is activated in cells of neural origin.

3.2 MATERIALS AND METHODS

3.2.1 Primers

human P1, 5'-GGAAGGAAGGGAATCACAG-3'

chick P1, 5'-GGAAAGGAGGGAAATCATCG-3'

human and chick P2, 5'-GGCACATCATAAACACCTTCC-3'

human and chick P3, 5'-GGAAGGTGTTTATGATGTGCC-3'

human and chick P4, 5'-ATGGGATAAAGGCATCACCT-3'

human P5, 5'-**ATG**TCAACTGAGACAGAACTTC-3'

human P5n, 5'-CTTCGAATTCT**ATG**TCAACTGAGACAGAACTTC-3'

human P6, 5'-TAT*CTA*GCTACCGGCCTG-3'

human P6n, 5'-GCAAGGATCCCTAT*CTA*GCTACCGGCCTG-3'

human actin (forward), 5'-CTGGCACCACACCTTCTAC-3'

human actin (reverse), 5'-CATACTCCTGCTTGCTGATC-3'

Eco RI (P5n) and *Bam* HI (P6n) restriction enzyme adapter sites are underlined. Start codons (P5 and P5n) are in bold. Stop codons (P6 and P6n) are in italics.

3.2.2 RT-PCR analysis

One μg of poly(A)$^+$ RNA from chick retina at day 5 of incubation (ED5), twelve human RB tumour cell lines, ten human NB tumour cell lines, and 4 μg of total RNA from human fetal retina (8 wks gestation), human fetal brain (8 wks gestation) and human RB tumour biopsies, were reverse-transcribed using an oligo d(T) primer and Superscript reverse transcriptase (Invitrogen). PCR amplification (35 cycles) was carried out using 1/20 of the cDNA generated from these reactions. Primer set P1 and P2 was used for the analysis of the two-exon region deleted in Dab1-E; primer set P3 and P4 was used for the analysis of the exon inserted in Dab1-E; and primer set P1 and P4 was used for the analysis of both the deletion and insertion regions (Figs. 3.1, 3.6, 3.7). Amplification of actin cDNA served as the positive control. DNA fragments were run in 10% polyacrylamide gels in Tris-borate EDTA (TBE) buffer. For sequencing, amplified cDNAs were either sequenced directly or cloned into the pCRII-TOPO TA-overhang cloning vector (Invitrogen) and sequenced.

For amplification of the entire human Dab1-E and Dab1-L open reading frames, a nested primer approach was used. Human 8 wk fetal retina cDNA was amplified with the P5/P6 primer set. Amplified cDNAs were purified and re-amplified with the P5n/P6n primer set containing *Eco*RI and *Bam*HI restriction enzyme sites (underlined in *Primers*), respectively. These cDNAs were cloned into pEGFP-C1 (BD Biosciences, San Jose), confirmed by PCR analysis and sequenced.

3.2.3 Transfection analysis

Transfection and confocal microscopy analysis of primary chick retina cultures were performed as previously described (Katyal and Godbout, 2004). Briefly, primary retinal cultures were prepared from trypsinized ED5 chick retinas plated onto glass coverslips (one-twelfth of a retina per 12 mm coverslip). Cells were transfected by calcium phosphate-mediated DNA precipitation and the DNA removed after 16 hrs. Thirty hrs later, the cells adhering to coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.5% Triton X-100/PBS and incubated with mouse monoclonal anti-phosphotyrosine antibody (1:250) (PT-66; Sigma) or mouse monoclonal anti-phospho-SFK^{Y416} antibody (1:50) (clone 9A6; Upstate, Charlottesville, VA) followed by Alexa 555 goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) (1:200). The coverslips were mounted on slides using glycerol containing 1 mg/ml *p*-phenylenediamine + 1 µg/ml 4', 6'-diamidino-2-phenylindole (DAPI) and viewed on a Zeiss LSM 510 confocal microscope.

For Reelin inhibition experiments, Dab1-L-tranfected retinal cells were treated with CR-50 antibody (kindly provided by Dr. T Curran, St. Jude Children's Research Hospital, TN). After the DNA was removed, 1 µl of CR-50 antibody was added to each well. After 24 hrs, another microliter of CR-50 was added for an additional 24 hrs. Cells were then fixed and stained with anti-phospho-SFK^{Y416} antibody. GFP-positive cells were detected by epifluorescence.

3.2.4 Immunofluorescence analysis of human fetal retina sections

Human fetal retinal tissue (8 wks and 13 wks gestation) were obtained from therapeutic abortions in accordance with guidelines specified by the Alberta Cancer Board Research Ethics Committee. Retinal tissue sections were prepared as previously described (Katyal and Godbout, 2004). Frozen sections were rehydrated in PBS, fixed in 4% PBS-buffered paraformaldehyde and permeabilized in 1% PBS-buffered NP-40. Sections were double-stained with rabbit polyclonal anti-Dab1 antibody (1:500) (Rockland Immunochemicals, Gilbertsville, PA) and either mouse anti-phospho-SFK^{Y416} antibody (1:25) or mouse anti-Reelin antibody (1:250) (clone 142; Calbiochem, San Diego, CA), followed by Alexa 555-conjugated goat anti-rabbit (for Dab1) and Alexa 488-conjugated goat anti-mouse (for phospho-SFK^{Y416} or Reelin) secondary antibodies, respectively (1:150). Sections were counterstained with the Hoescht 33258 fluorescent nuclear stain (Invitrogen) and mounted with Fluorosave (Calbiochem). Images were collected with a Zeiss-Axioplan II microscope (Carl Zeiss) equipped with a cooled charge-coupled device camera (Cooke Corporation).

3.2.5 Western blot analysis

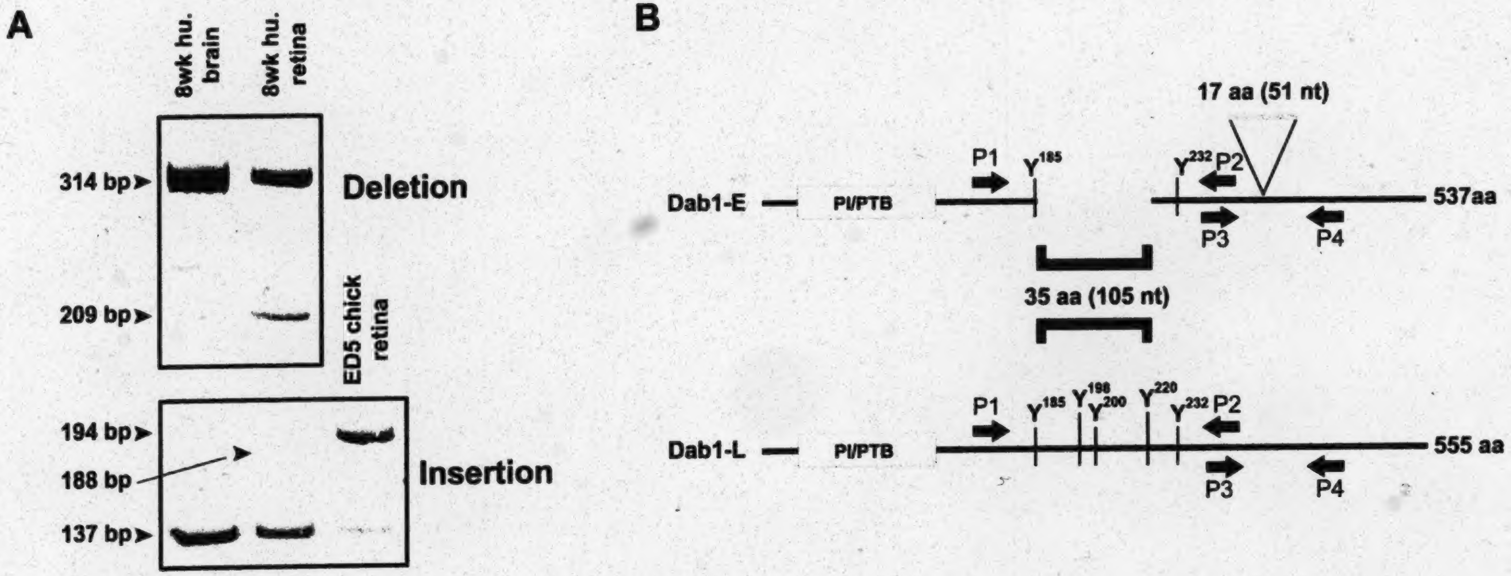
Whole cell lysates were prepared by mechanical disruption using a modified RIPA buffer supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride and 1X Complete (Roche) protease inhibitor cocktail. Forty micrograms of protein extract was electrophoresed through a SDS-7% poly-acrylamide gel (for Reelin) or a 10%-SDS poly-acrylamide gel (for Dab1). Extracts were subsequently electroblotted onto nitrocellulose followed by immunostaining with mouse anti-Reelin (1:500) or rabbit anti-Dab1 (1:1000) antibodies. Blots were immunolabelled with horseradish peroxidase-conjugated donkey anti-mouse (for Reelin) or donkey anti-rabbit (for Dab1) secondary antibodies (1:300000). Signals were visualized using the ECL Advance (GE Healthcare) chemiluminescence detection system.

3.3 RESULTS

3.3.1 Dab1 is alternatively spliced in the developing human fetal retina and brain

Based on our previous results, Dab1 is alternatively-spliced as a function of developmental age in chick retina (Katyal and Godbout, 2004). In contrast to Dab1-L which is expressed in amacrine and ganglion cells, Dab1-E is expressed in retinal progenitor cells, lacks two exons containing two SFK tyrosine phosphorylation sites and appears to function independent of events associated with SFK phosphorylation, Dab1-E also contains an extra 57 bp exon that is not found in Dab1-L. We used primers flanking the two-exon deletion region (P1, P2) and the insertion region (P3, P4) to see whether similar alternative splicing events also occurred in the human fetal retina. As shown in Fig. 3.1A, RT-PCR analysis of human fetal retina at 8 wks gestation using P1 and P2 primers generated two bands, of 314 bp and 209 bp, with the latter spanning the 105 bp two-exon (exons 7 and 8) deletion region. These two bands are of the same sizes as those obtained with ED5 chick retina cDNA, although the ratio of top band to bottom band differs between chicken and human. Similar results were obtained with human fetal brain at 8 wks gestation, except that the 209 bp band was barely detectable, suggesting that splicing events associated with the late form of Dab1 occur at an earlier developmental stage in the human brain than in retina, in keeping with our observations in chicken (Katyal and Godbout, 2004). Sequence analysis of the 314 bp and 209 bp bands verified that the top band contains the two-exon region specific to Dab1-L, while this region is excluded in the bottom band.

Fig. 3.1 - RT-PCR analysis of Dab1 deletion and insertion regions. (**A**) cDNAs synthesized from poly(A)$^+$ RNA from human fetal brain (8 wks gestation), retina (8 wks gestation) and chick retina (ED5) were amplified using P1 and P2 primers for deletion analysis and P3 and P4 primers for insertion analysis. Sizes of amplified bands are indicated. (**B**) Schematic of Dab1-E and Dab1-L protein and relative positions of primers used for RT-PCR amplification.


A
8wk hu. brain
8wk hu. retina
ED5 chick retina
314 bp
209 bp
Deletion
194 bp
188 bp
137 bp
Insertion
B
Dab1-E
PI/PTB
P1
Y185
Y232
P2
P3
P4
17 aa (51 nt)
537aa
35 aa (105 nt)
Dab1-L
PI/PTB
P1
Y185
Y198
Y200
Y220
Y232
P2
P3
P4
555 aa

RT-PCR analysis of human fetal retina using P3 and P4 primers generated two bands, of 188 bp and 137 bp, with the former predicted to encompass the insertion exon unique to Dab1-E. While clearly visible, the 188 bp band was considerably weaker than the 137 bp band. The 188 bp band was not detected in 8 wks human fetal brain. Sequence analysis of the 188 bp and 137 bp bands revealed an extra 51 bp in the top band, corresponding to the 57 bp exon previously identified in chick Dab1-E (Katyal and Godbout, 2004). Based on the published exon/intron structure of the human *Dab1* gene, this 51 bp exon corresponds to exon 9-2 (labeled 9B in Fig, 3.5), and has previously been shown to be alternatively spliced in embryonic mouse brain and chick eye (Bar *et al.*, 2003). The amino acid sequence of the two-exon deletion region (exons 7 and 8), the 51 bp insertion region (exon 9B) and sequence alignment of the region amplified in huDab1-E and huDab1-L by the P1/P4 primer set are indicated in Fig. 3.2.

3.3.2 Dab1 function is evolutionarily conserved in retina

To isolate full-length human Dab1-E and Dab1-L (huDab1-E and huDab1-L), human fetal retina cDNA was amplified using nested primers P5/P6 and P5n/P6n, with the latter containing *Eco*RI and *Bam*HI restriction enzyme sites, respectively (underlined in Primers – Materials and Methods). A total of twelve clones were obtained, one clone representing huDab1-E and eleven clones representing huDab1-L. Full-length huDab1-E and huDab1-L cDNAs, verified by sequencing, were cloned into pEGFP-C1. We expressed GFP-tagged huDab1-E

Fig. 3.2 - Sequence alignment of P1/P4-amplified Dab1 fragments. HuDab1-E, huDab1-L and chDab1 nucleotide and amino acid sequences are as indicated. The Dab1-L-specific 105 nt two-exon (7/8) region is indicated in blue while the huDab1-E-specific 51 nt exon 9B (57 nt for chDab1-E) is indicated in red. SFK (Y185 and Y198) and Abl/Crk (Y220 and Y232) recognition motifs are boxed.

```
             K  E  G  N  H  R  F  V  A  I  K  T  A  Q  A  A  E  P  V  I
huDab1-E    AAGGAAGGGAATCACAGATTTGTGGCCATAAAAACAGCCCAGGCGGCTGAACCTGTTATT  453
huDab1-L    AAGGAAGGGAATCACAGATTTGTGGCCATAAAAACAGCCCAGGCGGCTGAACCTGTTATT  453

                   P1

             L  D  L  R  D  L  F  Q  L  I  Y  E  L  K  Q  R  E  E  L  E
huDab1-E    CTGGACTTGAGAGATCTCTTTCAACTCATTTATGAATTGAAGCAAAGAGAAGAATTAGAA  513
huDab1-L    CTGGACTTGAGAGATCTCTTTCAACTCATTTATGAATTGAAGCAAAGAGAAGAATTAGAA  513

                                                         chDab1-L ->
             K  K  A  Q  K  D  K  Q  C  E  Q  A  V  Y  Q  T  I  L  E  E
chDab1      AAAAAGGCACAAAAGGACAAGCAGTGTGAACAGGCGGTATACCAGACAATTTTGGAAGAA
             K  K  A  Q  K  D  K  Q  C  E  Q  A  V  Y  Q  T  I  L  E  E
huDab1-E    AAAAAGGCACAAAAGGATAAGCAGTGTGAACAAGCTGTGTACCAG---------------  558
huDab1-L    AAAAAGGCACAAAAGGATAAGCAGTGTGAACAAGCTGTGTACCAGACAATATTGGAAGAG  573

             D  V  E  D  P  V  Y  Q  Y  I  V  F  E  A  G  H  E  P  I  R
chDab1-L    GATGTAGAAGACCCTGTATACCAGTACATTGTGTTTGAGGCTGGACATGAGCCAATCCGT
             D  V  E  D  P  V  Y  Q  Y  I  V  F  E  A  G  H  E  P  I  R
huDab1-E    ------------------------------------------------------------  558
huDab1-L    GATGTTGAAGATCCTGTGTACCAGTACATTGTGTTTGAGGCTGGACACGAGCCAATCCGT  633

                               <- chDab1-L
             E  P  E  T  E  E  N  I  Y  Q  V  P  T  S  Q  K  K  E  G  V
chDab1      GAGCCTGAAACAGAAGAAAACATTTATCAGGTTCCTACCAGCCAAAAGAAGGAAGGTGTT
             D  P  E  T  E  E  N  I  Y  Q  V  P  T  S  Q  K  K  E  G  V
huDab1-E    ------------------------------GTTCCCACCAGCCAAAAGAAGGAAGGTGTT  588
huDab1-L    GATCCCGAAACGGAAGAAAACATTTATCAGGTTCCCACCAGCCAAAAGAAGGAAGGTGTT  693

                                          chDab1-E ->
             Y  D  V  P  K  S  Q  P  V  S  L  E  N  G  N  L  L  L  D  I
chDab1      TATGATGTGCCAAAAAGTCAACCTGTAAGTCTGGAGAATGGAAACTTATTGCTGGACATT
             Y  D  V  P  K  S  Q  P  V  S  *  *  N  G  Y  S  F  E  D  F
huDab1-E    TATGATGTGCCAAAAAGTCAACCTGTAAGT******AATGGCTATTCGTTTGAGGATTTT  642
huDab1-L    TATGATGTGCCAAAAAGTCAACCTGTAAGT******------------------------  723

                            <- chDab1-E
             D  E  N  L  G  S  V  T  Q  A  V  T  Q  L  E  L  F  G  D  M
chDab1      GATGAAAATCTTGGTTCAGTCACTCAGGCTGTTACCCAACTAGAGCTTTTTGGGGACATG
             E  E  R  F  A  A  A  T  P  A  V  T  Q  L  E  L  F  G  D  M
huDab1-E    GAAGAACGGTTTGCTGCAGCCACCCCGGCTGTGACCCAATTAGAACTTTTTGGGGACATG  702
huDab1-L    ---------------------------GCTGTGACCCAATTAGAACTTTTTGGGGACATG  756

             S  T  P  P  D  I  T  S  P  P  T  P  A  T  P  G  D  A  F  I
huDab1-E    TCCACACCCCCTGATATAACCTCTCCCCCCACTCCTGCAACTCCAGGTGATGCCTTTATC  762
huDab1-L    TCCACACCCCCTGATATAACCTCTCCCCCCACTCCTGCAACTCCAGGTGATGCCTTTATC  816

                                                                P4
```

and huDab1-L in primary cultures of chick retina. As shown in Figs. 3.3C and 3.3D, and similar to results obtained with chicken Dab1-L, cells expressing human Dab1-L formed numerous thin elongated processes (Katyal and Godbout, 2004). In contrast, huDab1-E-expressing cells retained an undifferentiated epithelial-like appearance, similar to that obtained with GFP-transfected cells (Figs. 3.3A, 3.3B, and data not shown). Dab1-L-expressing cells also showed increased tyrosine phosphorylation (compare Fig. 3.3C with Fig. 3.3A) and SFK activation (compare Fig. 3.3D with Fig. 3.3B). These data suggest that the Dab1 functions that are related to SFK activation are conserved in human and chicken.

To determine whether Dab1-L-mediated SFK activation and associated morphological changes are Reelin-dependent, we treated GFP-Dab1-L transfected retinal cells with an anti-Reelin antibody, CR-50, previously shown to prevent Reelin dimerization and binding to receptors thereby abrogating Reelin-mediated signaling (Ogawa *et al.*, 1995; D'Arcangelo *et al.*, 1997). Dab1-L-expressing cells treated with CR-50 for two days showed a dramatic reduction in SFK activity compared to mock-treated Dab1-L-expressing cells (Figs. 3.4A, 3.4B). Furthermore, cells treated with CR-50 had a round, undifferentiated morphology reminiscent of Dab1-E-expressing cells. These data indicate that Reelin signaling is required for Dab1-L function in retinal cultures.

Fig. 3.3 - Analysis of Dab1-transfected primary retinal cultures. (**A** and **C**) GFP expression and phosphotyrosine (pTyr) immunoreactivity in GFP-huDab1 transfected retinal cultures. (**B** and **D**) GFP and phosphoSFK^{Y416} (pSFK) expression in GFP-huDab1 transfected retinal cultures. Cells were fixed with 4% paraformaldehyde, permeabilized and stained with either anti-phosphotyrosine or anti-phosphoSFK^{Y416} antibodies followed by Alexa 555-conjugated goat anti-mouse secondary antibody. The GFP signal in transfected cells was detected by epifluorescence.

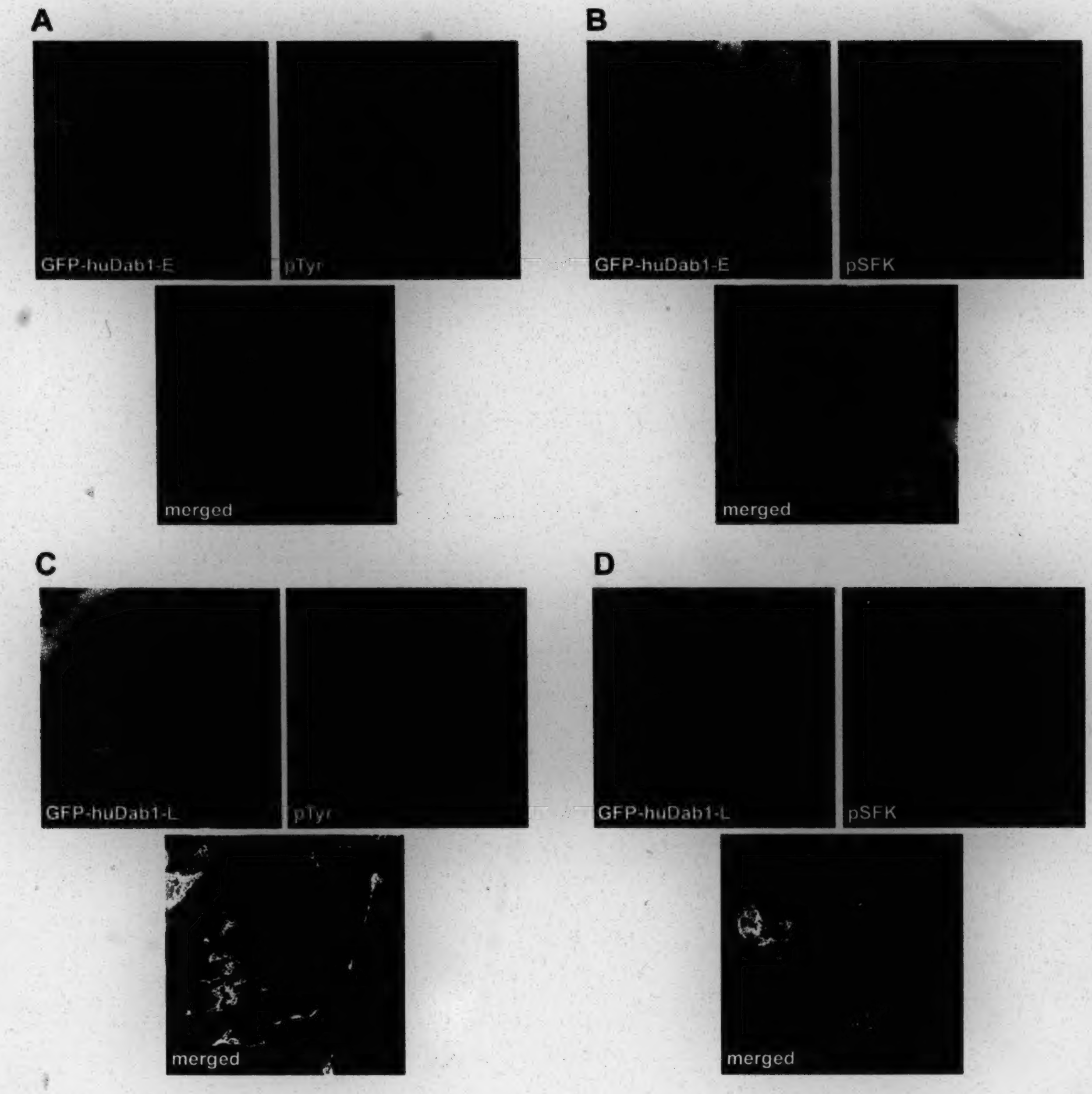
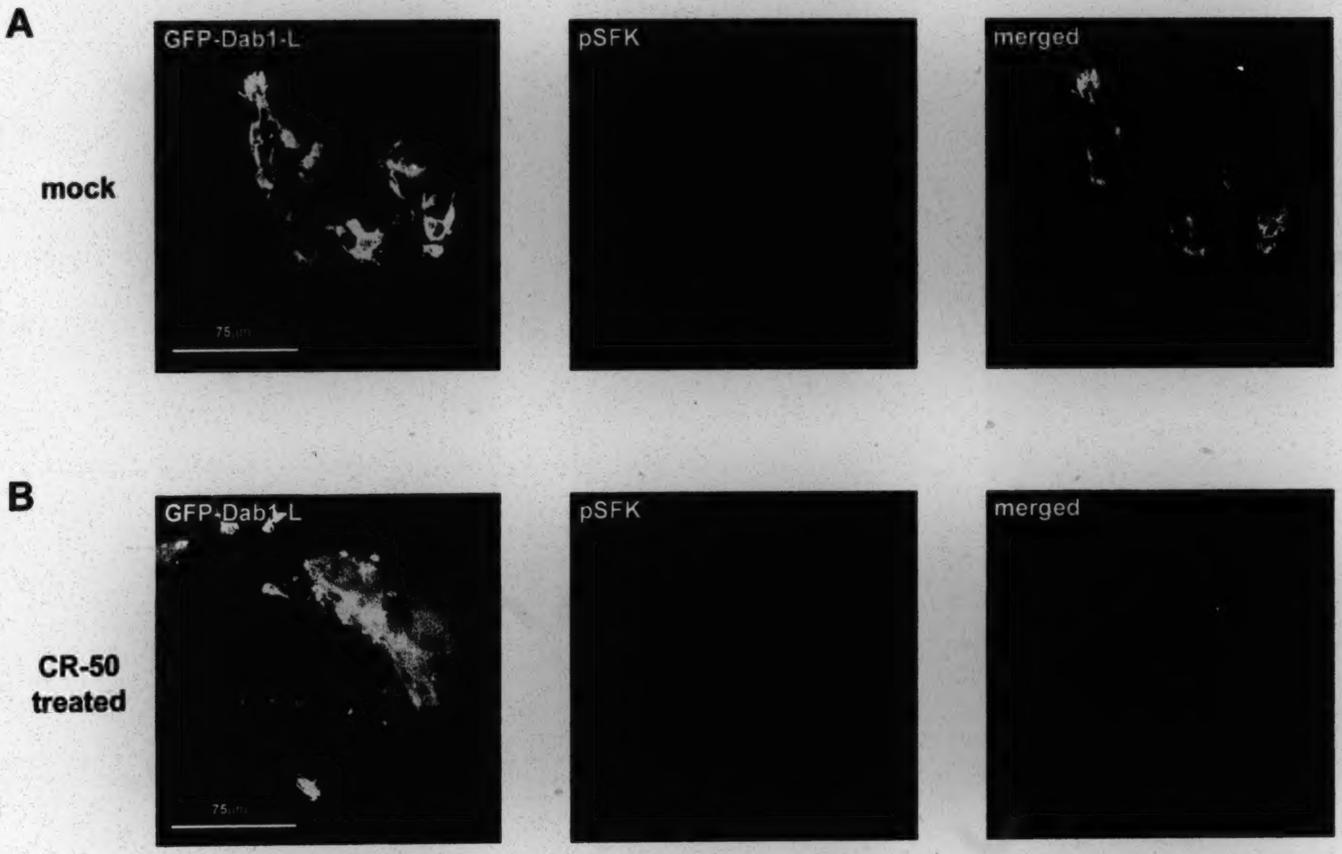

A
GFP-huDab1-E
pTyr
merged
B
GFP-huDab1-E
pSFK
merged
C
GFP-huDab1-L
pTyr
merged
D
GFP-huDab1-L
pSFK
merged

Fig. 3.4 - Analysis of CR-50-treated Dab1-L-expressing retinal cells. GFP-huDab1-L and phospho-SFK^{416} (pSFK) expression in mock- (**A**) and CR-50-treated (**B**) cells. Sixteen hours after DNA transfection, cells were washed and exposed to two sequencial treatments of 1 μl/ml CR-50 (24 hours for each treatment). Cells were fixed with 4% paraformaldehyde, permeabilized and stained with anti-phosphoSFK^{Y416} antibody followed by Alexa 555-conjugated goat anti-mouse secondary antibody. The GFP signal in transfected cells was detected by epifluorescence.


A
mock
GFP-Dab1-L
pSFK
merged
75 µm
B
CR-50
treated
GFP-Dab1-L
pSFK
merged
75 µm

3.3.3 Dab1 expression correlates with Reelin expression and SFK activation in the developing human retina

Frozen human fetal retina tissue sections at 8 and 13 wks gestation were double-stained with anti-Dab1 (red) and either anti-Reelin or anti-phospho-SFK^{Y416} antibodies (green). Analysis of the 8 wk retina revealed weak Dab1 immunostaining in the undifferentiated inner neuroblastic layer (INBL) and stronger staining of the ganglion cell layer (GCL) (Fig. 3.5A). Both Reelin (data not shown) and activated SFK (Fig. 3.5A) had a similar pattern, with highest levels in the GCL and ganglion nerve fiber layer. By 13 wks gestation, the Dab1 signal was much more pronounced in the GCL compared to the inner nuclear layer (INL), although the emerging outer nuclear layer (ONL) was also positive (Figs. 3.5B). Dab1-positive cells were positive for both Reelin (data not shown) and activated SFKs (Fig. 3.5B).

3.3.4 Retinoblastoma and neuroblastoma tumour cells express multiple alternatively-spliced Dab1 transcripts

RB tumours have been postulated to be derived from a neuronal precursor cell committed to the amacrine and/or horizontal lineage (Jiang *et al.*, 1997; Robanus-Maandag *et al.*, 1998; Gallie *et al.*, 1999; Chen *et al.*, 2004). NB tumours are believed to be derived from neural crest neuroectodermal precursor cells (Westermann and Schwab, 2002). To investigate whether either Dab1-E or -L is expressed in these tumours, we carried out RT-PCR analysis of 12 RB tumour cell lines and 10 NB cell lines. As shown in Fig. 3.6, Dab1 products were

Fig. 3.5 - Immunofluorescence analysis of Dab1 (red) and phospho-SFK^{Y416} (green) in the developing human retina. Sections were double-stained with anti-Dab1 and anti-phospho-SFK^{Y416} (pSFK) antibodies followed by Alexa 555-conjugated donkey anti-rabbit and Alexa 488-conjugated donkey anti-mouse secondary antibodies. Sections were counterstained with the fluorescent dye Hoescht 33258 to label the nuclei. Retinal tissue sections were prepared from human fetal retina at 8 wks gestation (**A**) and 13 wks gestation (**B**). Abbreviations are: RPE, retinal pigment epithelium; INBL, inner neuroblastic layer; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

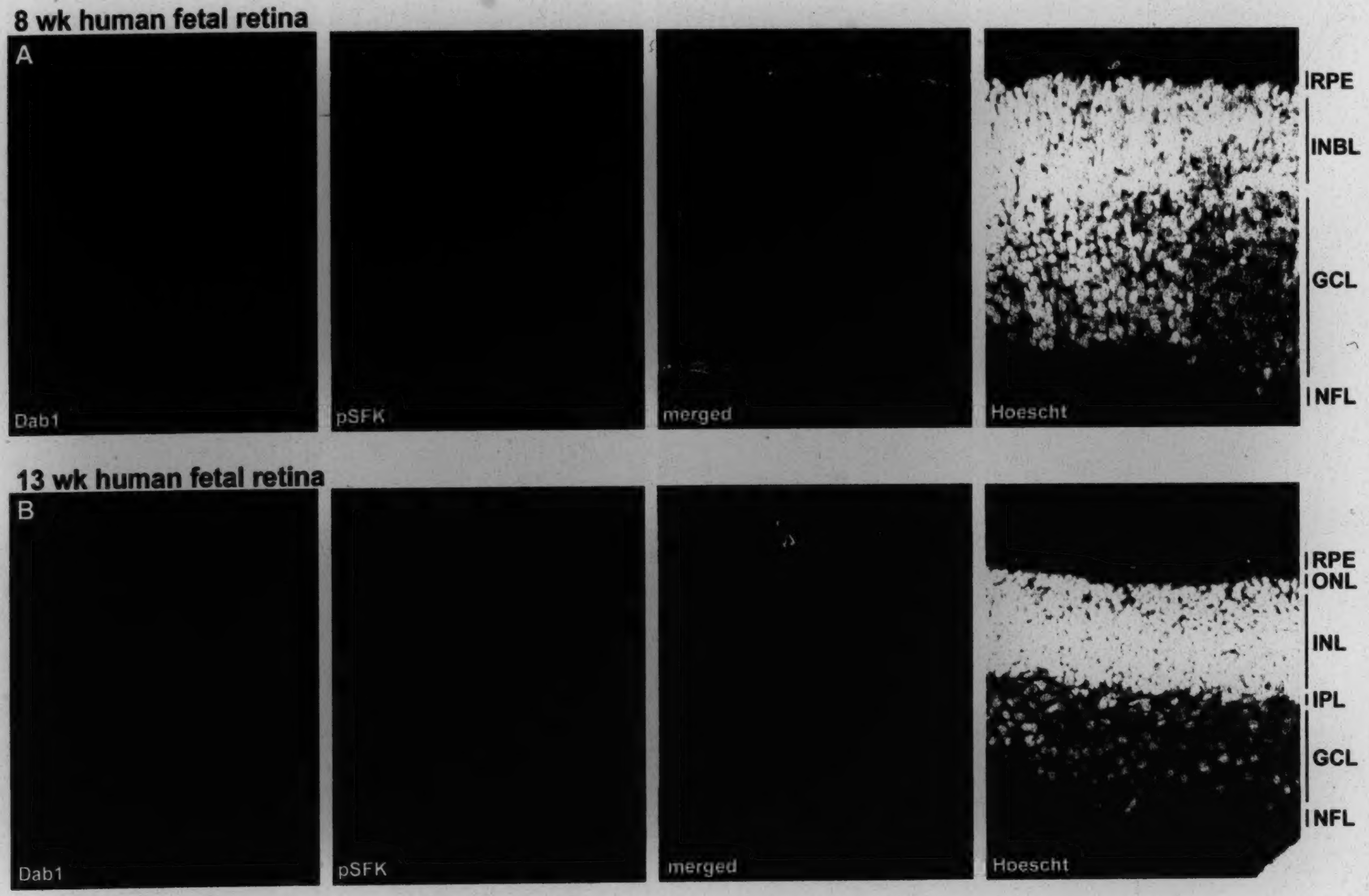
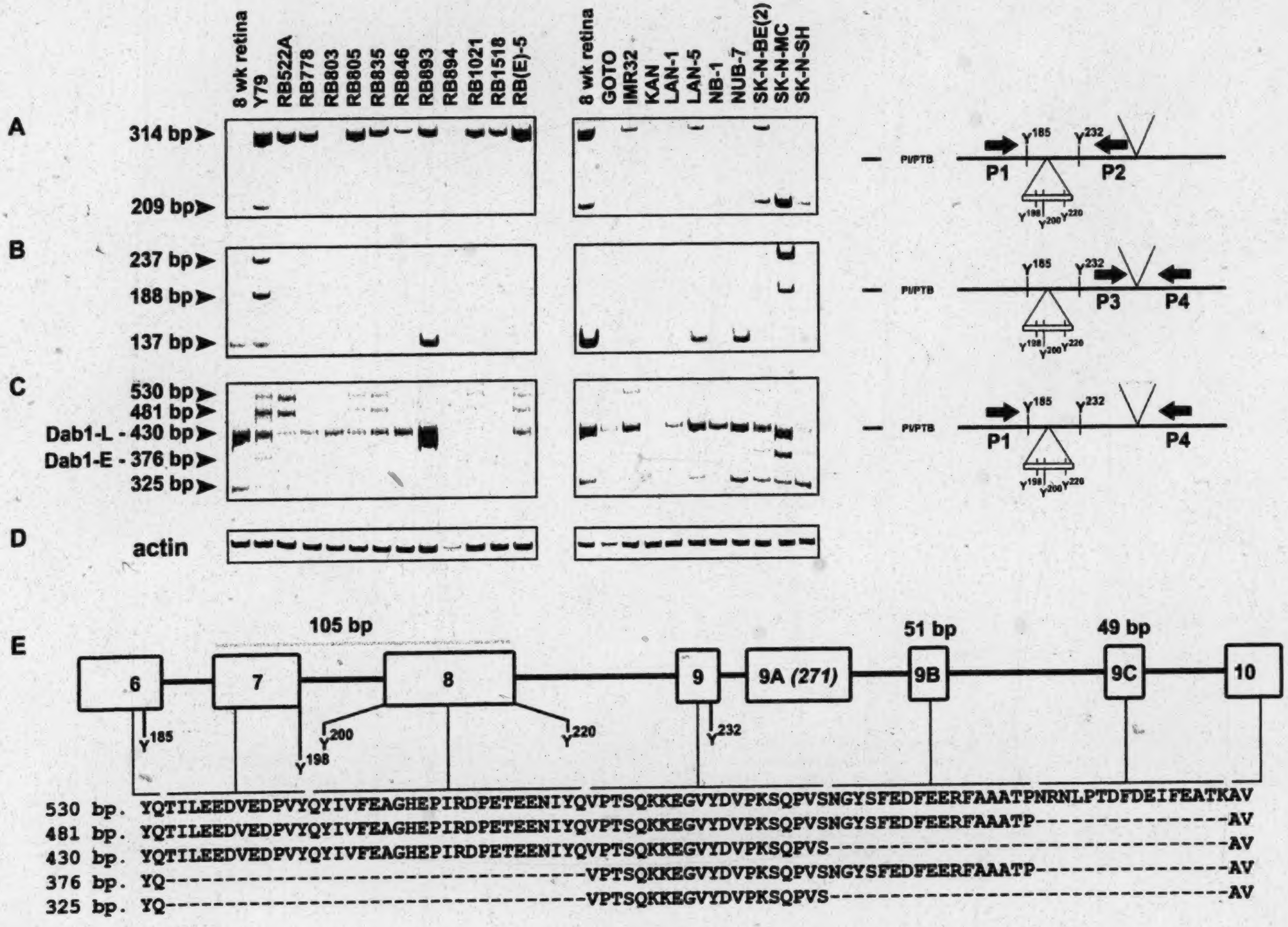

8 wk human fetal retina
A
Dab1
pSFK
merged
Hoescht
RPE
INBL
GCL
NFL
13 wk human fetal retina
B
Dab1
pSFK
merged
Hoescht
RPE
ONL
INL
IPL
GCL
NFL

Fig. 3.6 - Identification of *Dab1* splice forms expressed in RB and NB cell lines. (**A**) The region corresponding to exons 7/8 containing SFK and Abl/Crk recognition motifs was amplified from tumour cDNAs using primer set P1/P2. (**B**) The region corresponding to exons 9/9B/9C was amplified using primer set P3/P4. (**C**) Amplification of the combined regions amplified in **A** and **B** using primer set P1/P4. (**D**) Amplification of actin cDNA to control for variation in the amount of RNA used. (**E**) Alignment of Dab1 sequences corresponding to *Dab1* splice forms expressed in RB and NB using primer set P1/P4. A schematic representation of the human *Dab1* genomic intron/exon structure spanning exon 6 to exon 10 is shown. The amino acid sequence encoded by these exons is as indicated for the sequence of the 530 bp *Dab1* cDNA amplified in **C**. The size and sequences of the four other fragments amplified in **C** are as indicated and aligned to show exon inclusion/exclusion in each *Dab1* splice form.


A
B
C
D
E
314 bp
209 bp
237 bp
188 bp
137 bp
530 bp
481 bp
Dab1-L - 430 bp
Dab1-E - 376 bp
325 bp
actin
8 wk retina
Y79
RB522A
RB778
RB803
RB805
RB835
RB846
RB893
RB894
RB1021
RB1518
RB(E)-5
8 wk retina
GOTO
IMR32
KAN
LAN-1
LAN-5
NB-1
NUB-7
SK-N-BE(2)
SK-N-MC
SK-N-SH
PI/PTB
P1
P2
P3
P4
Y185
Y198
Y200
Y220
Y232
105 bp
51 bp
49 bp
6
7
8
9
9A (271)
9B
9C
10
530 bp. YQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVSNGYSFEDFEERFAAATPNRNLPTDFDEIFEATKAV
481 bp. YQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVSNGYSFEDFEERFAAATP-----------------AV
430 bp. YQTILEEDVEDPVYQYIVFEAGHEPIRDPETEENIYQVPTSQKKEGVYDVPKSQPVS----------------------------------AV
376 bp. YQ----------------------------------VPTSQKKEGVYDVPKSQPVSNGYSFEDFEERFAAATP-----------------AV
325 bp. YQ----------------------------------VPTSQKKEGVYDVPKSQPVS----------------------------------AV

observed in all RB and NB tumour cell lines, although there was significant variation in relative band intensities depending on primer set and cell line used. Using the P1 and P2 primers, two bands were observed: a 314 bp band specific to Dab1-L which includes exons 7 and 8 and a 209 bp band specific to Dab1-E that lacks these exons (Fig. 3.6A). The 314 bp band was predominant in the great majority of RB tumour lines as well as in 2 NB cell lines (IMR32, LAN-5). The 209 bp band was predominant in SK-N-MC and SK-N-SH. With the P3 and P4 primers, three bands were observed, of 237 bp, 188 bp and 137 bp (Fig. 3.6B). The 188 bp band contains exon 9B, characteristic of Dab1-E, while the 137 bp band lacks this exon, characteristic of Dab1-L. The 237 bp band represents a splice variant that was previously documented by Bar *et al.* (2003) and contains exon 9C as well as exon 9B sequences (Fig. 3.6E). All three bands were similarly amplified in RB cell lines with the exception of RB778 and RB893, which showed stronger levels of the 137 bp band compared to the 188 bp and 236 bp bands. LAN-5, Nub-7 and SK-N-SH NB lines also had a more intense 137 bp band.

Results obtained with P1/P2 and P3/P4 primer sets suggest that: (i) splice variants in addition to the previously characterized Dab1-E and -L are present in RB and NB cells, and (ii) there is not a direct correlation between deletion of exons 7 and 8 and insertion of exon 9B (and vice versa) in the tumour cell lines, in contrast to what has been reported in chick retina (Katyal and Godbout, 2004). To address the spectra of alternatively spliced products in RB and NB lines, we used the P1 and P4 primers to amplify the entire region spanning the insertion/deletion region. Five major products were observed, all of which are clearly depicted in the Y79 lane (Fig. 3.6C). Based on sequence analysis: (i) the 530 bp band includes exons 7, 8, 9, 9B and 9C, (ii) the 481 bp band includes exons 7, 8, 9 and 9B, (iii) the 430 bp band, corresponding to Dab1-L, includes exons 7, 8 and 9, (iv) the 376 bp band, corresponding to Dab1-E, includes exons 9 and 9B, and (v) the 325 bp band includes only exon 9 (Fig. 3.6E). Of note, the 430 bp band, representing Dab1-L, was present in relatively high abundance in the majority of RB and NB cell lines. Higher molecular weight products containing exons 7/8/9/9B or exons 7/8/9/9B/9C were predominant in RB lines, while lower molecular products excluding exons 7/8/9B or exons 7/8/9B/9C were predominantly found in NB cell lines. These results suggest that splicing is controlled differently in RB and NB cells, perhaps reflecting the nature of the *trans*-acting splicing factors expressed in these two tumours. Interestingly, the 325 bp DNA fragment which excludes exons 7/8/9B/9C, was relatively abundant in human fetal retina. The counterpart to this DNA band was not observed in chick retina at any of the developmental stages tested, suggesting that this may represent an intermediate splice product or a final splice product specific to human.

To address whether alternative splicing of Dab1 might be affected by growth of tumour cells in culture, RT-PCR analysis with the P1/P4 primer set was carried out using total RNA isolated from two RB tumour biopsies. The corresponding cell lines were included for comparison. A remarkable level of similarity, both at the quantitative and qualitative levels, was observed between matching tumours and cell lines. As previously noted, the 430 bp band, representing Dab1-L, was predominant in both the tumours and their corresponding cell lines (Fig. 3.7). The 481 bp and 530 bp bands were also present in both sets of tumours and cell lines. These data indicate that Dab1 splicing and Dab1 function in RB cells have not been significantly altered as the result of growth in tissue culture, in keeping with the observation that RB cell lines appear stable in culture, with few karyotypic and morphological changes (Gallie *et al.*, 1982; Bogenmann and Mark, 1983). A similar analysis could not be carried out with NB tumours as no fresh tumour material with corresponding cell line was available.

3.3.5 Analysis of Reelin and Dab1 protein levels in RB and NB cell lines

To address whether the Reelin-Dab1 signaling pathway is active in RB and NB cell lines, we prepared whole cell extracts from three RB (RB522A, RB805 and RB835) cell lines and one NB (IMR32) cell line and carried out western blot analysis with anti-Reelin and anti-Dab1 antibodies. As shown in Fig. 3.8, only one RB cell line, RB805, expressed detectable Reelin and Dab1. Levels of Reelin and Dab1 in this cell line were considerably lower than those observed in ED7 chick retina. The Reelin band detected in RB805 and ED7 chick retina was 180 kDa. Activation of Reelin requires its proteolytic processing into smaller fragments, one of which is 180 kDa (Tissir and Goffinet, 2003), suggesting that activated Reelin is produced in this cell line.

Fig. 3.7 - Comparison of Dab1 splice forms expressed in two RB cell lines and their corresponding primary tumours. cDNAs derived from RB tumours and cell lines were amplified using primer set P1/P4. The size of the amplified bands is as indicated.

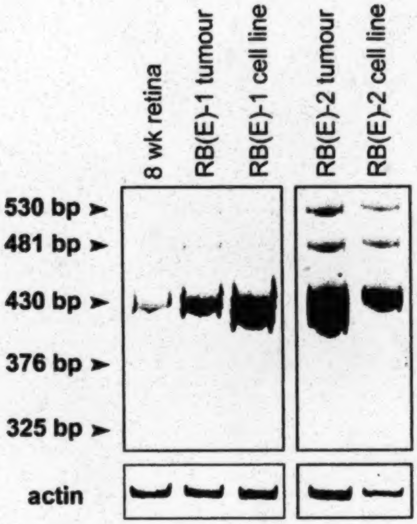
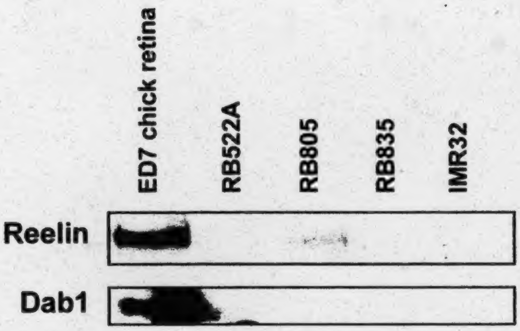

8 wk retina
RB(E)-1 tumour
RB(E)-1 cell line
RB(E)-2 tumour
RB(E)-2 cell line
530 bp ➤
481 bp ➤
430 bp ➤
376 bp ➤
325 bp ➤
actin

Fig. 3.8 - Western blot analysis of RB and NB tumour cell lines. Forty micrograms of protein extract were electrophoresed through a 7% polyacrylamide-SDS gel (for Reelin) or 10% polyacrylamide-SDS gel (for Dab1) and electroblotted onto nitrocellulose. Blots were immunostained with mouse anti-Reelin (1:500) and rabbit anti-Dab1 (1:1000) antibodies, respectively, followed by labeling with appropriate HRP-conjugated secondary antibodies.


ED7 chick retina
RB522A
RB805
RB835
IMR32
Reelin
Dab1

3.4 DISCUSSION

In this study we demonstrate that the two alternatively-spliced forms of Disabled-1, Dab1-E and Dab1-L, previously identified in embryonic chick retina are also present in human fetal retina. Like chDab1-E, expression of huDab1-E occurs early in the retina, at a time when retinal cells are undergoing proliferation. Likewise, similar to chDab1-L, huDab1-L is expressed at later stages of retinal development, when retinal subtypes form and cell lamination occurs. Transfection of huDab1-L into primary chick retinal cultures results in the formation of neurite-like processes, enhanced phosphotyrosine levels and activation of SFKs, while cells expressing huDab1-E show round undifferentiated cell morphology and do not display increased phosphotyrosine levels or SFK activation. Our results indicate that Dab1-E and Dab1-L have evolutionarily conserved functions in chick and human retinal development.

A key difference between Dab1-E and Dab1-L is the loss of two exons containing two SFK recognition motifs in Dab1-E while two Abl/Crk recognition motifs are retained in both Dab1 forms. One of these SFK motifs encompasses tyrosine-198, an essential residue for Reelin-induced tyrosine phosphorylation of Dab1 (Keshvara *et al.*, 2001). A second difference is the inclusion of a 51 bp exon (exon 9B) in Dab1-E as compared to Dab1-L. A similar exon is included in chDab1-E; however, chick *Dab1* exon 9B is 57 nt instead of 51 nt. To date, there is no known motif or function associated with exon 9B in human or chick. In chick, we have previously shown that the switch from *chDab1-E* to *chDab1-L* is mutually exclusive and occurs without any RNA splice intermediate, with a simultaneous "splicing in" of exons 7/8 and "splicing out" of exon 9B (Katyal and Godbout, 2004). This suggests that the splicing factors involved in these splicing events are highly coordinated. However, in human retina, we have detected a *Dab1* splice intermediate lacking both exons 7/8 and 9B in addition to the *Dab1-E* and *Dab1-L* forms. This intermediate was amplified to a higher level than that of Dab1-E using human fetal retina cDNA at 8 wks gestation and the P1/P4 primer set. The presence of this intermediate may suggest a specific requirement for the double-deleted Dab1 form during human retinal development. Alternatively, the splicing events associated with Dab1 isoform switching in human retina may not be as well-coordinated as those found in chick. Under this scenario, this human-specific splice intermediate would likely arise from the uncoordinated expression of the splicing factors required for human Dab1 isoform switching as the strong sequence similarity observed between human and chick *Dab1s* likely precludes differences in RNA splicing regulatory elements. A third explanation for our observations may relate to the nature of the human retinal tissue used in these experiments. Ready access to chick embryos allows us to dissect and process retinas within a fraction of the time that it takes to process human fetal retinas. As a result, we are able to capture a snapshot of the mRNA transcripts and proteins present at specific stages of embryonic chick retinal development. Conversely, the lag time between human retina delivery and processing could result in a variety of RNA artifacts arising from their extended presence in a suboptimal environment (Ars *et al.*, 2000; Messiaen *et al.*, 2000). If the double-deleted Dab1 form is as abundant as our RT-PCR results suggest, we would have expected to isolate this form when we cloned Dab1 cDNAs for expression analysis. Instead, none of the 12 clones isolated corresponded to double-deleted *Dab1* (11 were Dab1-L and 1 was Dab1-E). These results suggest that the human double-deleted Dab1 intermediate is not a full-length Dab1 splice form and may represent an RNA artifact.

Splice site selection during RNA splicing requires the combined recognition of a 5'-end splice donor site (MAGgu) and a downstream 3'-end splice acceptor site (cagG) (Black, 2003; Kalnina *et al.*, 2005). Site selection is regulated by adjacent *cis*-regulatory RNA elements which are bound by *trans*-acting splicing protein factors. *Trans*-acting factors include serine-arginine (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) which can have splicing enhancing and splicing suppressive functions, respectively. Alternative splicing arises from the alternative recognition of 5'-end and 3'-end splice sites due to differing types, combinations or concentrations of *trans*-acting factors that bind these *cis*-regulatory elements. Expression and/or repression of these *trans*-acting splice factors have been shown to be involved in developmental stage-specific alternative splicing of a variety of genes. For example, developmentally-regulated alternative splicing of the neurotransmitter receptor, glutamate receptor-2 subunit (*GluR2*), has been documented during cortical maturation (Crovato and Egebjerg, 2005). As development progresses, the early "flip" *GluR2* isoform containing the "flip" exon (exon 15) is spliced out and replaced by the adjacently located "flop" exon (exon 14) resulting in expression of the later "flop" *GluR2* isoform. These two isoforms differ in pharmacological and kinetic properties associated with neurotransmitter desensitization (Sommer *et al.*, 1990; Koike *et al.*, 2000). Mutually exclusive flip-flop isoform switching is regulated by two distinct SR-proteins, ASF/SF2 and SC35, which bind to splice enhancer elements located within the flop exon. Although we do not know which *trans*-acting splicing factors are involved in Dab1 alternative splicing, our study is one of only a few to provide a physiological role for development stage-specific alternative splicing.

Using our unique assay, we have identified a biochemical function for both isoforms in Reelin-mediated signaling. In our previous study, cells expressing either chDab1-E, which lacks Reelin-responsive Y198, or a chDab1-L^{Y198F} mutant did not tyrosine phosphorylate Dab1, activate SFKs or induce process formation (Katyal and Godbout, 2004). Here we show that either expression of huDab1-E or inhibition of Reelin-receptor binding using the CR-50 antibody, results in identical phenotypes (ie. lack of SFK activation and no process formation). Human (tissue sections) and chick retina (cultures) immunofluorescence studies combined with our CR-50 experiments clearly demonstrate that Reelin is expressed at a time when Dab1-E is also expressed (human 8 wk retina and ED5 primary chick retina cultures) and is consistent with previously published reports of Reelin expression in the developing chick retina (Bernier *et al.*, 2000).

The presence of Dab1-E expression in the developing human and chick retina points to a novel mechanism designed to prevent premature Reelin signaling during early stages of retinal development. At early stages, there are many proliferating cells as well as some differentiating cells primarily committed to the ganglion cell lineage. Thus, cellular proliferation, migration and differentiation occur simultaneously in the early retina. We propose that retinal progenitor cells expressing Dab1-E function independently of Reelin, while cells which have undergone Dab1 isoform switching, express Reelin-responsive Dab1-L. Expression of Dab1-L recouples the Reelin-Dab1 pathway thereby allowing these cells to undergo Reelin-dependent migration and neuronal differentiation.

Although another group has previously documented Dab1 expression in amacrine cells of newborn and adult human retina (Lee *et al.*, 2004), this is the first study to examine expression of Reelin-Dab1 signaling components in the human fetal retina. We found that at 8 wks gestation, Reelin, Dab1 and activated SFK are co-expressed throughout the inner neuroblastic layer, while at later stages (13 wks) these proteins are co-expressed in the emerging ganglion cell and nerve fiber layers. In chick, we have previously shown Dab1 to be expressed in all cell types of the early retina [embryonic day(ED) 5] while at later stages (ED16), Dab1 is localized to amacrine cells and the GCL (Katyal and Godbout, 2004). We have also found that Reelin is co-expressed with Dab1 in the ED16 retina (our unpublished data) while activated SFKs are found at higher levels within amacrine cells and the GCL compared to other cell types (Katyal and Godbout, 2004). In mouse, Rice and Curran (2001) have previously reported Dab1 expression in type AII amacrine cells in the P18 retina; however, examination of retinas derived from earlier mice (P3 and P7) show clear but weak Dab1 expression in the inner neuroblastic layer and ganglion cell layer in addition to amacrine cells (Rice and Curran, 2000; Rice *et al.*, 2001). Based on these data we conclude that the retinal patterns of Dab1 expression are similar in chick, mouse and human.

As the Reelin-Dab1 signaling pathway is important in neurogenesis, we also sought to characterize this pathway in two tumours of neuroectodermal lineage: RB and NB. Using RT-PCR, we show that Dab1 alternative splicing is deregulated in these tumours as indicated by the identification of up to five alternative *Dab1* isoforms differing by inclusion or exclusion of adjacent exons 8, 9, 9B and 9C. Comparison of RNAs derived from tumour biopsies with corresponding cultured tumour cells exclude the possibility that these isoforms are artifacts of prolonged growth in culture. In all tumour cell lines, we found that *Dab1-L* is the most predominant form expressed; however, expression levels of the other Dab1 alternatively-spliced forms varied between RB and NB with a predominance of higher molecular weight *Dab1* forms in RB and lower molecular weight *Dab1* forms in NB. Overall, *Dab1* transcripts appeared to be in low abundance as 35 cycles of PCR amplification produced minimal amounts of amplified cDNA. Furthermore, Dab1 and Reelin were only deleted in one out of four tumour cell lines tested by western blot analysis. Reelin and Dab1 protein levels in this cell line were dramatically reduced when compared to embryonic retina. These results are consistent with the Reelin-Dab1 pathway not playing a prominent role in these tumours. It is interesting to postulate that the absence or low abundance of Dab1 protein in RB and NB cell lines reflects a high rate of *Dab1* RNA degradation, likely the result of aberrant of inefficient RNA splicing

(Gudikote *et al.*, 2005). Reelin has also been documented to undergo alternative splicing (Lambert de Rouvroit *et al.*, 1999). It will be informative to determine whether multiple Reelin splice forms also exist in RB and NB and to correlate Reelin transcript levels with Reelin protein expression in these tumours. Loss of this pathway in tumours of neuroectodermal origin is significant considering its role in neuronal development. Formation of RB and NB tumours is a result of hyperproliferation of deregulated cells. Normally, in neuronal development, cells committed to differentiation lose their proliferative ability. Therefore, tumours derived from cells of neuronal origin must inactivate or perturb pro-differentiation signals, such as those transduced by the Reelin-Dab1 pathway. In agreement with this concept, it is interesting to note that the RB cell line (RB805) that has minimal Reelin/Dab1 protein expression displays a slow rate of growth in culture (our unpublished data).

The presence of multiple Dab1 splice products in RB and NB indicates that the activities of splicing factors may be deregulated in these tumours. Aberrant splicing is a common property of tumours (Venables, 2004). At least 15% of all point mutations associated with human disease occur in *cis*-regulatory RNA elements and result in splicing defects (Krawczak *et al.*, 1992). In cancer, these mutations can dramatically alter alternative splicing of specific proto-oncogenes and tumour suppressors resulting in dramatic changes in their biochemical function. For example, many neurofibromas arise from mutations of the *NF1* gene. Some of these mutations result in the formation of a variety of *NF1* splice forms, characterized by exon skipping and/or alternative usage of 5'-

and 3'-end splice sites (Serra *et al.*, 2001). Another cause for alternative splicing in tumours is the deregulated activity of *trans*-acting splicing factors. For example, the p210 BCR/ABL mutation associated with chronic myelogenous leukemia (CML) induces the expression of a variety of genes involved in pre-mRNA splicing, including RNA helicase 2/Gu, hnRNP A2/B1 and SF3B (Salesse *et al.*, 2004). Expression of these proteins correlates with changes in splicing efficiency of the non-receptor tyrosine kinase PYK2. In addition to changes in protein sequences, alternative splicing can also alter RNA activity, including subcellular localization, translation potential and RNA degradation (Mignone *et al.*, 2002). Our observation that different levels and types of Dab1 splice forms exist in RB and NB may be attributed to any number of factors. One reason may be related to the tumorigenic event underlying RB and NB. RB tumours are characterized by inactivation of *RB* while the more aggressive form of NB are often accompanied by *MYCN* amplification (Brodeur, 1990; Schwab, 1990; Schweigerer *et al.*, 1990). Both these genes have been shown to be involved directly or indirectly in gene transcription. Any alterations to their activity will have profound effects on the spectrum of genes expressed in these tumours. Therefore, it is possible that among the genes differentially expressed in these tumours, some may be involved in alternative splicing of Dab1.

In summary, we have isolated the human splice forms of *Dab1-E* and *Dab1-L* from the developing human retina. We have found that the expression and function of the two isoforms are evolutionarily conserved in human and chick retina. These results point to a conserved mechanism for the regulation of premature Reelin signaling early in neuronal development. We have also found that *Dab1* transcripts are expressed in two tumours of neuroectodermal cell origin: RB and NB. In these tumours, Dab1 alternative splicing is deregulated as multiple forms of Dab1, in addition to those found in retina, are expressed. Furthermore, Reelin and Dab1 protein levels are either absent or drastically reduced in these tumours. These results point to a requirement for inactivation of the Reelin-Dab1 pathway in RB and NB. Future work will include Reelin treatment combined with Dab1 transfection analysis of RB and NB to determine if reactivation of this pathway has any affect on tumour growth and properties.

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Chapter 4

Molecular analysis of Dab1 tyrosine phosphorylation and protein interactions

Running title: *Dab1 tyrosine phosphorylation and protein interactions*

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4.1 INTRODUCTION

Brain development requires the migration and correct positioning of cortical neurons into distinct cell layers. The Reelin-Disabled-1 pathway is believed to be critical to the regulation of cortical lamination. Reelin, an extracellular glycoprotein, is secreted by specific neuronal cell types within the developing cortex: Cajal-Retzius and GABAergic neurons (D'Arcangelo *et al.*, 1995; Impagnatiello *et al.*, 1998). Reelin binds to its receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), typically expressed by adjacently located pyramidal neurons and migrating cortical neurons (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Tissir and Goffinet, 2003). Reelin signaling results in receptor clustering and membrane recruitment of Disabled-1 (Dab1) (Strasser *et al.*, 2004). Dab1 is a cytosolic adaptor protein which undergoes Reelin-induced activation when it binds to cytoplasmic FxNPxY docking sites located on the intracellular tails of VLDLR and ApoER2 (Hiesberger *et al.*, 1999; Howell *et al.*, 1999a; Howell *et al.*, 1999b). Reelin-mediated receptor clustering induces Dab1 oligomerization and basal phosphorylation at tyrosine-198. In turn, tyrosine phosphorylated Dab1 binds to and mediates phosphorylation-dependent activation of Src-family kinases (SFK), including Fyn and Src (Keshvara *et al.*, 2001; Bock and Herz, 2003; Arnaud *et al.*, 2003b; Strasser *et al.*, 2004). Activated Fyn has been reported to induce Dab1 hyperphosphorylation at tyrosines-198, -220 and -232 (Ballif *et al.*, 2004), suggesting that Dab1 and Fyn form a mutually self-regulating association. Dab1 tyrosine phosphorylation is critical to the transduction of the Reelin signal as mice that express a mutant form of Dab1 lacking all candidate tyrosine phosphorylation sites (*Dab1*^{5F}) show identical neuronal positioning defects to those seen in *Reelin*$^{-/-}$ (*reeler*), *Dab1*$^{-/-}$ (*scrambler/yotari*) and *VLDLR*$^{-/-}$*ApoER2*$^{-/-}$ mutant mice; these mice are characterized by ataxia, cerebellar hypoplasia and inversion of neuronal layering/cortical lamination (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Ware *et al.*, 1997; D'Arcangelo *et al.*, 1999; Trommsdorff *et al.*, 1999; Howell *et al.*, 2000). Tyrosine phosphorylated Dab1 has been shown to recruit a number of SH2-containing proteins implicated in actin- and microtubule-mediated cytoskeletal reorganization and neuronal migration. Specific Dab1 tyrosine phosphorylated sites bind specific SH2-containing proteins; e.g. proteins associated with Dab1 phosphotyrosine-198 include SFKs (Fyn, Src, Yes), phosphoinositol 3'-kinase (PI3K) and phospholipase C-gamma1 (PLCγ1), while those associated with Dab1 phosphotyrosine-220/232 include the Crk family of adaptor proteins (CrkI, CrkII, CrkL, Nckα and Nckβ) (Howell *et al.*, 1997; Beffert *et al.*, 2002; Suetsugu *et al.*, 2002; Ballif *et al.*, 2003; Pramatarova *et al.*, 2003; Ballif *et al.*, 2004; Suetsugu *et al.*, 2004). A recent study shows that the dynein/dynactin-associated protein, LIS1, interacts with Dab1 phosphorylated at both tyrosine-198 and tyrosine-220 (Assadi *et al.*, 2003). LIS1 is involved in microtubule-dependent transport, neuronal migration and axonal outgrowth (Smith *et al.*, 2000). These differing and overlapping phosphotyrosine-dependent interactions suggest that Dab1 tyrosine phosphorylation may be coordinated and interrelated in such a way as to mediate downstream cytoskeletal-modulating and cell migratory signals.

We have previously shown that expression of full-length Dab1 (Dab1-L) in cultured retinal neurons induces the formation of thin elongated GAP-43-positive processes, reminiscent of neurites associated with differentiation (Katyal and Godbout, 2004). This effect is dependent on Dab1 phosphorylation at tyrosine-198 and activation of SFKs, as cells that express a Dab1^{Y198F} mutant protein fail to form processes and do not show induction of phosphotyrosine. In contrast, cells that express Dab1 mutated at three other tyrosine residues (Y185, Y200 or Y220) behave normally. Tyrosine-185 and tyrosine-198 are part of SFK recognition motifs (Y-Q-x-I) while tyrosine-220 and another tyrosine (Y232) that was not tested, are part of Abl-family kinase/Crk recognition motifs (Y-x-V-P).

Through analysis of retinal and brain tissue at early stages of development, we discovered an alternatively-spliced variant of Dab1, called Dab1-early (Dab1-E) which lacks two exons containing tyrosine-185 and tyrosine-198 phosphorylation sites. We found that there was a correlation between Dab1-E expression, reduced phosphotyrosine, lack of SFK activation and reduced GAP-43 levels in retinal tissue at early stages of development. As the retina matures, Dab1-L is expressed in two retinal subtypes, amacrine and ganglion, accompanied by increased phosphotyrosine levels, activation of SFKs and increased levels of GAP-43. These results indicate that retinal precursor cells undergo isoform switching as they differentiate into specific neuronal cell lineages, suggesting that Dab1-E is required prior to neuritogenesis, while Dab1-L, with accompanying phosphorylation at tyrosine-198 and possible tyrosine-185 is critical for functions related to differentiation. As Dab1 phosphorylated at tyrosine-198 has been shown to mediate Reelin-Dab1 signaling, we further propose that only Dab1-L responds to Reelin.

To further delineate the contribution of tyrosine phosphorylation to Reelin-mediated Dab1 signaling, we have performed a detailed mutational analysis of all key Dab1 tyrosine phosphorylation sites (Y185, Y198, Y220, Y232) singly and in combination. These Dab1 mutants were expressed in primary chick retinal cultures and analyzed for levels of phosphotyrosine, SFK activation and changes in cellular morphology/process formation. The second component of this chapter examines the role of the protein tyrosine phosphatase SHP-2, previously shown to interact with Dab1, in the regulation of Reelin-Dab1 signaling. In the third component of this chapter, we have used the yeast two-hybrid system to identify proteins interacting with Dab1-E and Dab1-L. Our study provides new insight into the molecular mechanisms that regulate Dab1 and Dab1-dependent activity during neuronal development.

4.2 MATERIALS AND METHODS

4.2.1 Generation of Dab1-L mutants

Generation of site-directed single, double and triple Dab1 Y→F mutants was carried out by sequential PCR (Cormack and Castano, 2002). Partially complementary primers containing a point mutation corresponding to a tyr → phe substitution [TA(T/C) → TT(T/C)] were used in conjunction with pEGFP-C1 vector primers located upstream of the *Eco*RI site and downstream of the *Bam*HI site to generate DNA fragments corresponding to full-length chDab1-L, each mutated at a specific tyrosine residue. DNA fragments were annealed, extended and amplified using pEGFP-C1 vector primers. The DNA was digested with *Eco*RI and *Bam*HI and cloned into pEGFP-C1. Constructs were sequenced to ensure that they were error-free. Expression of full-length GFP-chDab1-L mutant proteins was confirmed by transfection and western blot analysis. Double mutants were generated from corresponding single mutants while triple mutants were generated from corresponding double mutants.

Chicken Dab1^{Y185F}, Dab1^{Y198F}, Dab1^{Y220F} and Dab1$^{Y185F/Y198F}$ mutants have been previously described (Katyal and Godbout, 2004). In total, nine new mutants were generated: Dab1^{Y232F}, Dab1$^{Y185F/Y220F}$, Dab1$^{Y198F/Y220F}$, Dab1$^{Y185F/Y232F}$, Dab1$^{Y198F/Y232F}$, Dab1$^{Y220F/Y232F}$, Dab1$^{Y185F/Y198F/Y220F}$, Dab1$^{Y185F/Y198F/Y232F}$, Dab1$^{Y185F/Y220F/Y232F}$ and Dab1$^{Y198F/Y220F/Y232F}$. Primers used to generate these mutants have also been previously described (Katyal and Godbout, 2004), with the exception of those specific for Dab1^{Y232F} (sense strand, 5'-AGGTGTTTTTGATGTGCCA-3'; antisense strand, 5'-ACATCAAAAACACCTTCCTT-3').

4.2.2 Transfection analysis

Primary retinal cultures were prepared from ED5 chick retinas trypsinized prior to plating onto glass coverslips (one-twelfth of a retina per 12 mm coverslip). Cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum and incubated in a 5% CO_2 humidified chamber. For transfection analysis of chDab1-L mutants, 1 µg of DNA was transfected per well, while for transfection analysis of Dab1 and SHP-2, a 1:1 ratio of pEGFP-huDab1-L (or pEGFP-huDab1-E) (described in Chapter 3) and pRK5-SHP-2 (or pRK5 control) (kindly provided by D. Burshtyn, Univ. of Alberta) was used per well (for a total of 1 μg per well). The DNA was introduced into cells by calcium phosphate-mediated DNA precipitation and the DNA removed after 16 hrs. Thirty hrs later, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 mins and permeabilized for 5 mins in 0.5% Triton X-100/PBS. Cells were incubated overnight with mouse anti-phosphotyrosine (PT-66) (1:250) (Sigma) or mouse anti-phospho-Src$^{(Y416)}$ (9A6) (1:50) (Upstate) antibodies followed by Alexa 555-conjugated goat anti-mouse secondary antibody (Molecular Probes) (1:200) for one hr. The coverslips were mounted on slides using glycerol containing 1 mg/ml *p*-phenylenediamine + 1 μg/ml 4', 6'-diamidino-2-phenylindole (DAPI). Cells were viewed on a Zeiss LSM 510 confocal microscope.

4.2.3 Yeast two-hybrid analysis

N-terminal fragments of huDab1-E (aa 1-257) and huDab1-L (aa 1-273) were generated by PCR amplification using primers P4 and P5 (described in Chapter 2). These fragments were ligated in-frame with the GAL4 binding domain (BD) within the *Bam*HI site of the Matchmaker Two-Hybrid System III (BD Biosciences, San Jose) bait vector, pGBKT7-BD. Two-hybrid screening was performed using the sequential transformation protocol, where the bait plasmids are first transformed into yeast cells, followed by the prey library. pGBKT7-Dab1-

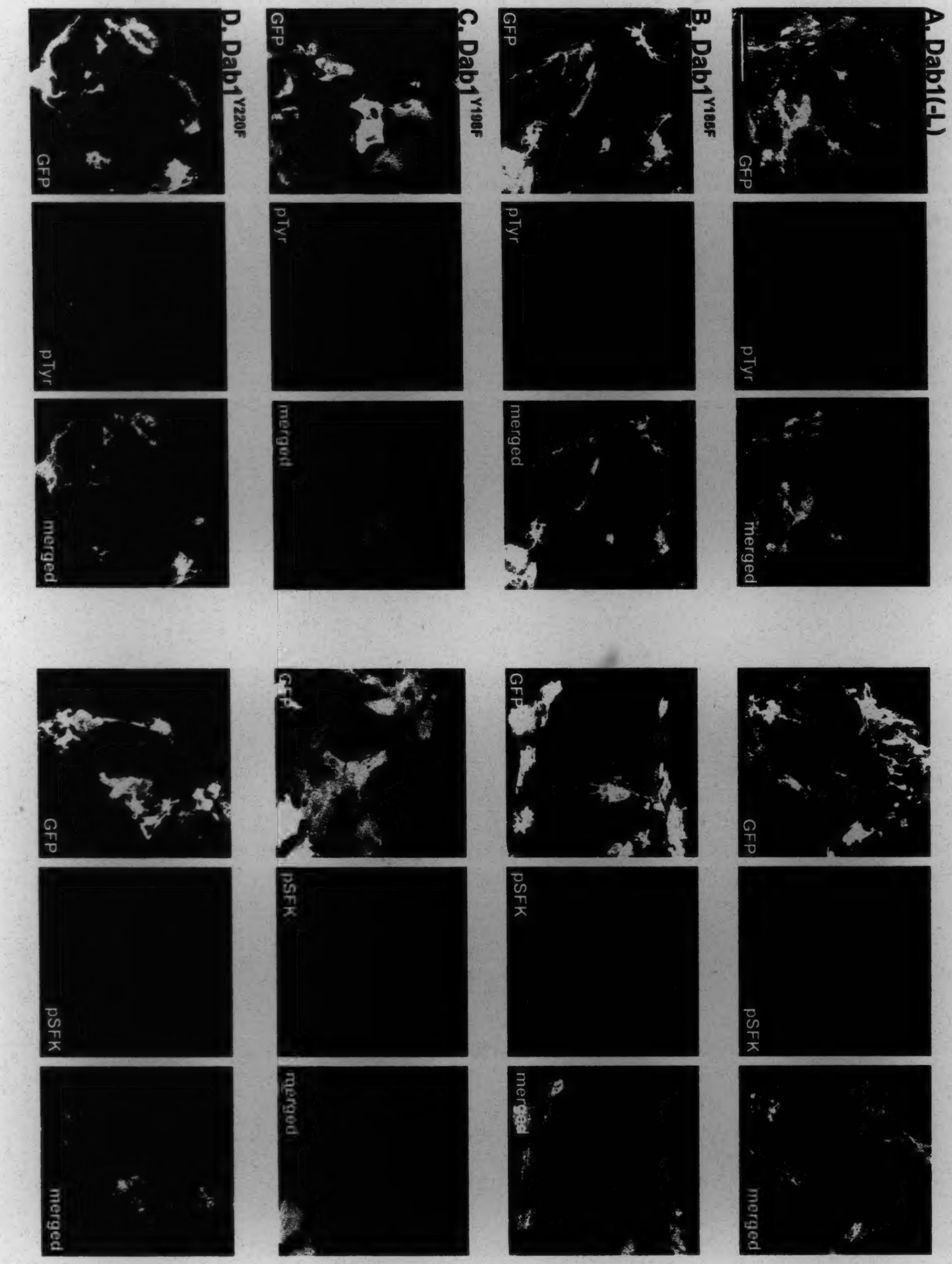
E or -L were transformed into the AH109 (auxotrophic for tryptophan, leucine, histidine and adenine) yeast strain and grown for 3 days on synthetic dropout medium lacking tryptophan (SD/-trp) to select for yeast cells expressing the *TRP1* gene present in pGBKT7. Cells were also plated on medium lacking both tryptophan and histidine (SD/-trp/-his) to confirm inability of the BD-Dab1 fusion protein to self-activate the HIS3 nutritional reporter and grow in the absence of histidine. Large scale sequential library transformation was then performed according to the manufacturer's protocol. Briefly, BD-Dab1-E- or BD-Dab1-L-expressing AH109 yeast cells were transformed with 30 ug of a human fetal brain cDNA library fused with the GAL4 activating domain (AD) in the pACT2 yeast expression vector (BD Biosciences, San Jose). Transformed yeast cells were grown on medium lacking tryptophan (described earlier), leucine (which selects for AH109 cells transformed with the pACT2-library prey construct which expresses LEU2), histidine (HIS3 nutritional reporter driven by the GAL1 UAS promoter; described earlier) and adenine (ADE2 nutritional reporter driven by the GAL2 UAS promoter which produces yeast with a cream/pale pink colour) (SD/-trp/-leu/-his/-ade). This medium was also supplemented with 5 mM 3-amino-1,2,4 triazole (3-AT) (Sigma-Aldrich, St. Louis), a competitive inhibitor for the HIS3 protein resulting in increased stringency of the library screen. Co-transformed colonies were allowed to grow for 7-10 days prior to their selection for further analysis. Colonies were selected on the basis of colour (cream to light pink), size (1 mm to 5 mm), morphology (completely round with no indentations or kinks) and protrusion from the media surface (ie. not flat). Colonies that met these criteria were restreaked on SD/-trp/-leu/-his/-ade media, then on SD/-trp/-leu medium (to segregate false-positive colonies arising from mutiply-transformed prey constructs), then again on SD/-trp/-leu/-his/-ade medium. Colonies that survived re-streaking were then subjected to beta-galactosidase colony-lift filter assays to determine whether the putative BD-Dab1/AD-protein interaction could activate a third reporter, lacZ (driven by the MEL1 UAS promoter). Prey plasmid DNA was isolated from yeast colonies that turned blue. These plasmids were subsequently re-transformed into both BD-Dab1-E and BD-Dab1-L expressing AH109 cells to confirm the two-hybrid interaction. Prey plasmids corresponding to these positive colonies were then sequenced with the T7 sequencing primer and underwent BLAST analysis to determine identity.

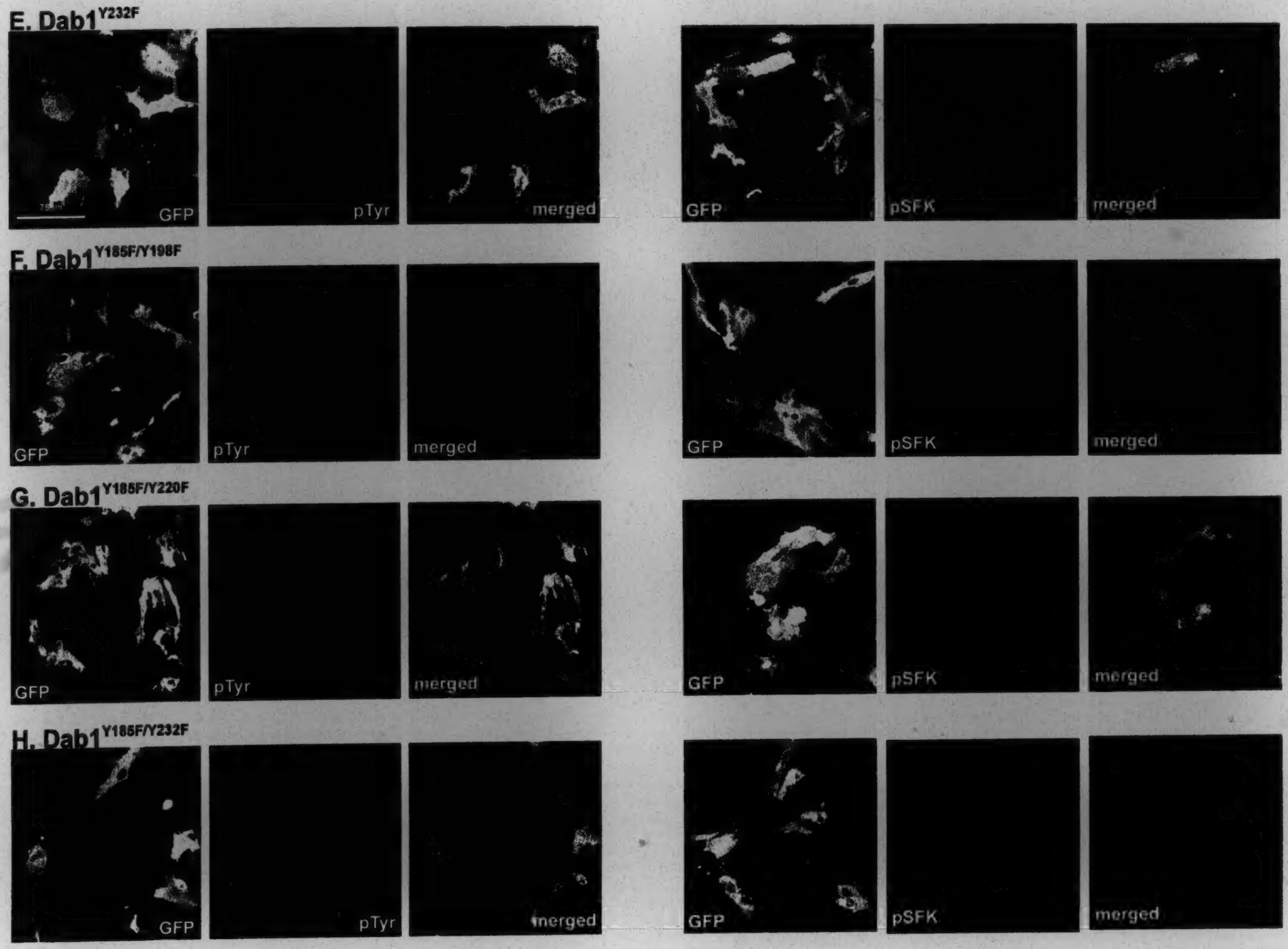
4.3 RESULTS

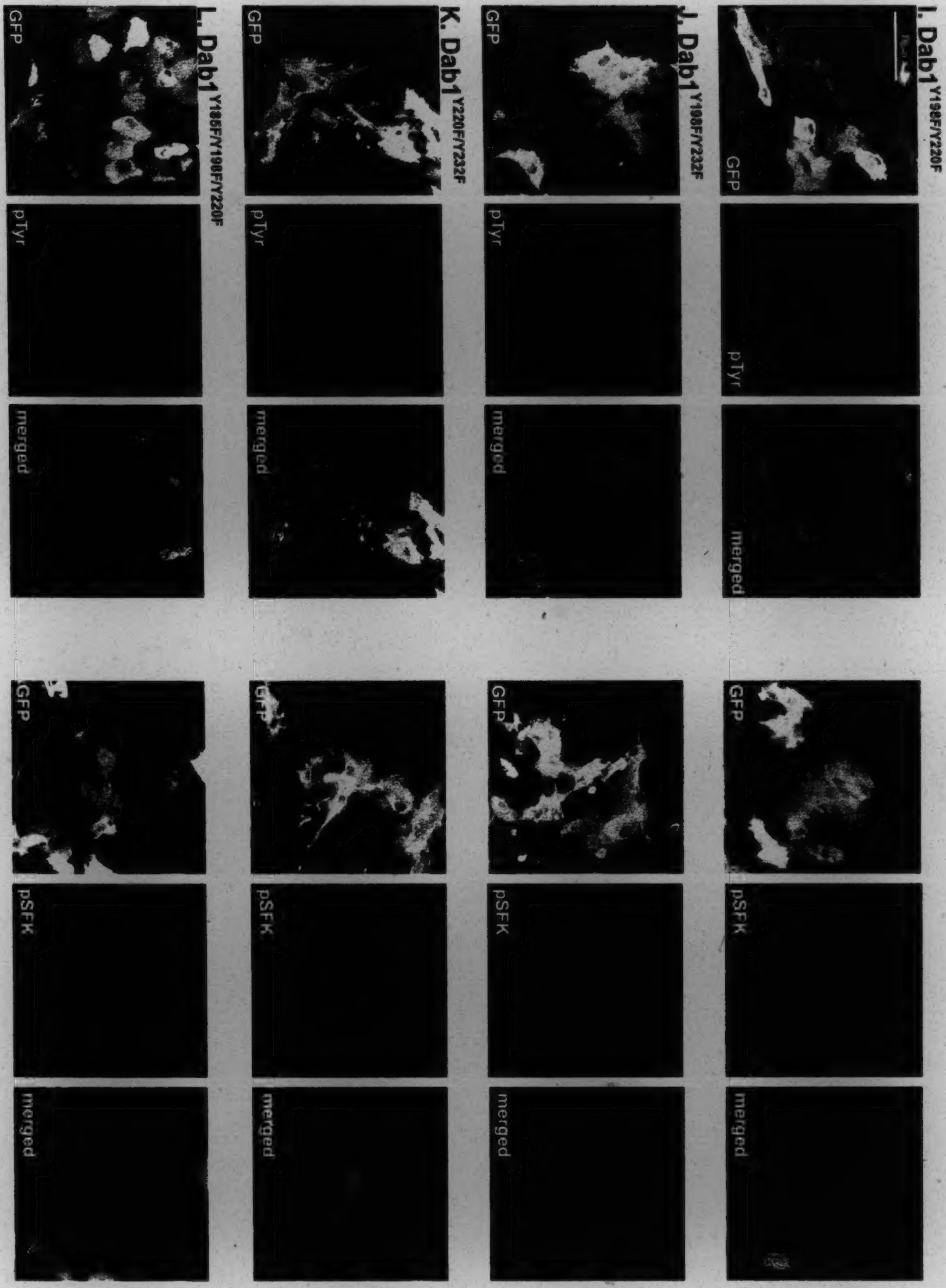
4.3.1 Reelin-Dab1-mediated neurite formation depends on multiple Dab1 tyrosine phosphorylation sites

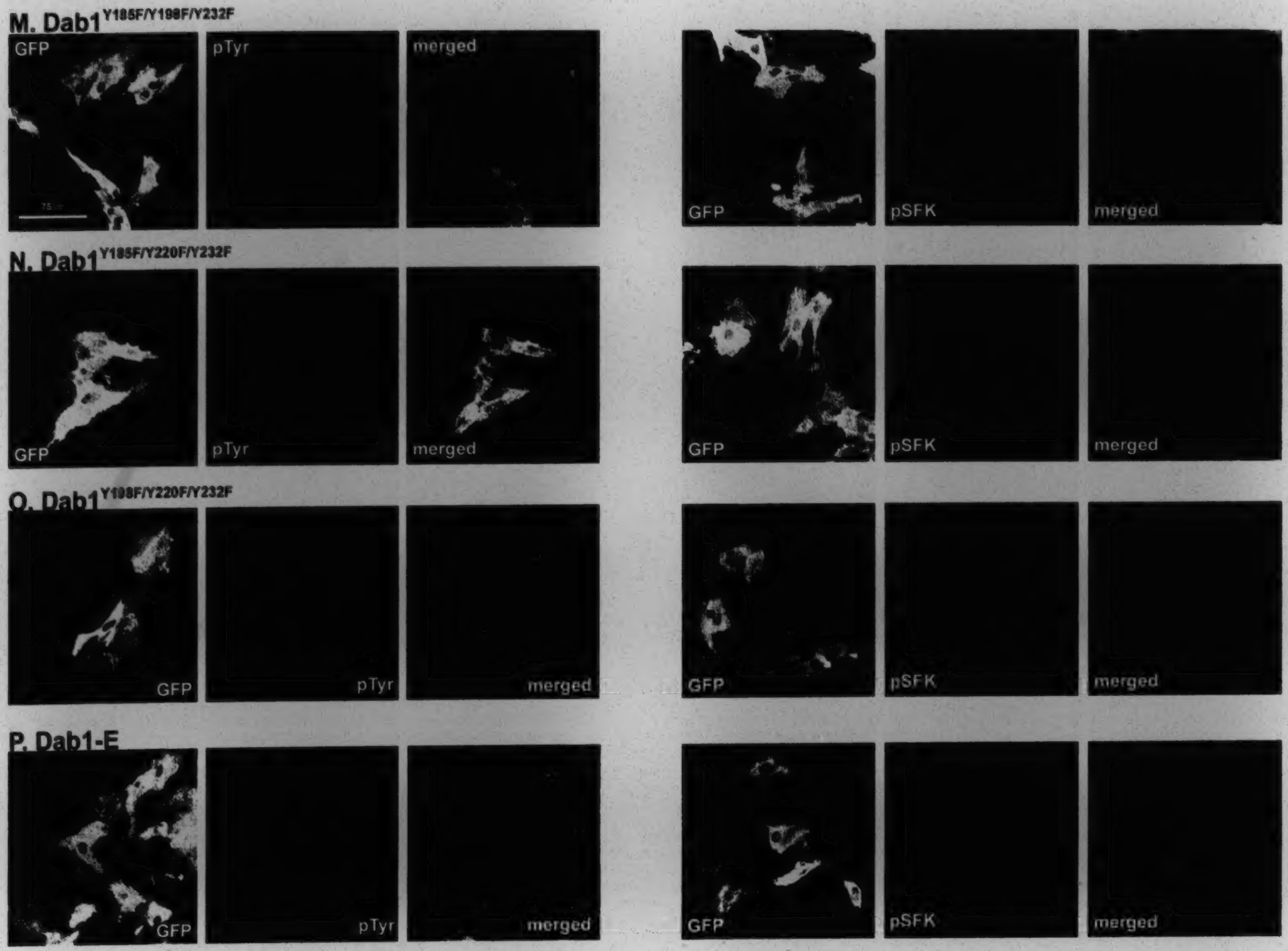
Previous Dab1 tyrosine phosphorylation mapping studies identified tyrosine-198 as the first (and main) residue to undergo Reelin-mediated phosphorylation while tyrosine-220 was identified as a secondary, less phosphorylated residue (Keshvara *et al.*, 2001). More recent studies have shown that Reelin signaling results in phosphorylation at tyrosine-198, tyrosine-220 and tyrosine-232 (Ballif *et al.*, 2004; Chen *et al.*, 2004; Huang *et al.*, 2004). These observations suggest the possibility of hierarchical phosphorylations of Dab1 upon Reelin stimulation. In order to study possible coordination or interrelationship between phosphorylation at tyrosines 185, 198, 220 and 232, we generated a series of GFP-Dab1-L constructs harboring combinations of single, double and triple Y→F substitutions. These mutant constructs were transfected into primary chick retinal cultures, fixed and fluorescently stained with antibodies to phosphotyrosine and phosphoSFK^{Y416}. Cellular morphology, phosphotyrosine levels and SFK activation were documented for each construct.

As previously reported, retinal cells expressing Dab1^{Y198F} had an undifferentiated epithelial-like morphology, showed little phosphotyrosine immunoreactivity and no SFK activation (Fig. 4.1C), similar to Dab1-E-expressing cells (Fig. 4.1P) (Katyal and Godbout, 2004). In contrast, the morphology and properties of cells expressing Dab1^{Y185F} (Fig. 4.1B) and Dab1^{Y220F} (Fig. 4.1D) mutants were virtually identical to that of cells expressing wild-type Dab1-L (Fig. 4.1A); ie. strong phosphotyrosine immunoreactivity, SFK activation and the formation of numerous thin elongated neurite-like processes (Katyal and Godbout, 2004). Interestingly, cells expressing Dab1^{Y232F} displayed morphology that was neither Dab1-E-like nor Dab1-L-like, but rather resembled a combination of both (Figs. 4.1E). Similar to Dab1-L, cells expressing Dab1^{Y232F} showed increased levels of phosphotyrosine; however, in contrast to Dab1-L, Dab1^{Y232F}-expressing cells showed dramatically reduced activation of SFK, similar to Dab1-E-expressing cells (compare Figs. 4.1A, 4.1P with Fig. 4.1E). These data suggest that Dab1 tyrosine-198 is primarily responsible for Reelin-mediated Dab1 tyrosine phosphorylation, induction of SFKs and associated cell cytoskeletal/morphological events, while tyrosine-232 is required for SFK


A. Dab1(-L)
B. Dab1^{Y185F}
C. Dab1^{Y198F}
D. Dab1^{Y220F}
GFP
pTyr
merged
GFP
pSFK
merged


E. Dab1Y232F
75 µm
GFP
pTyr
merged
GFP
pSFK
merged
F. Dab1Y185F/Y198F
GFP
pTyr
merged
GFP
pSFK
merged
G. Dab1Y185F/Y220F
GFP
pTyr
merged
GFP
pSFK
merged
H. Dab1Y185F/Y232F
GFP
pTyr
merged
GFP
pSFK
merged


I. Dab1$^{Y198F/Y220F}$
J. Dab1$^{Y198F/Y232F}$
K. Dab1$^{Y220F/Y232F}$
L. Dab1$^{Y185F/Y198F/Y220F}$
GFP
pTyr
merged
GFP
pSFK
merged


M. Dab1$^{Y185F/Y198F/Y232F}$
N. Dab1$^{Y185F/Y220F/Y232F}$
O. Dab1$^{Y198F/Y220F/Y232F}$
P. Dab1-E
GFP
pTyr
merged
pSFK
75 µm

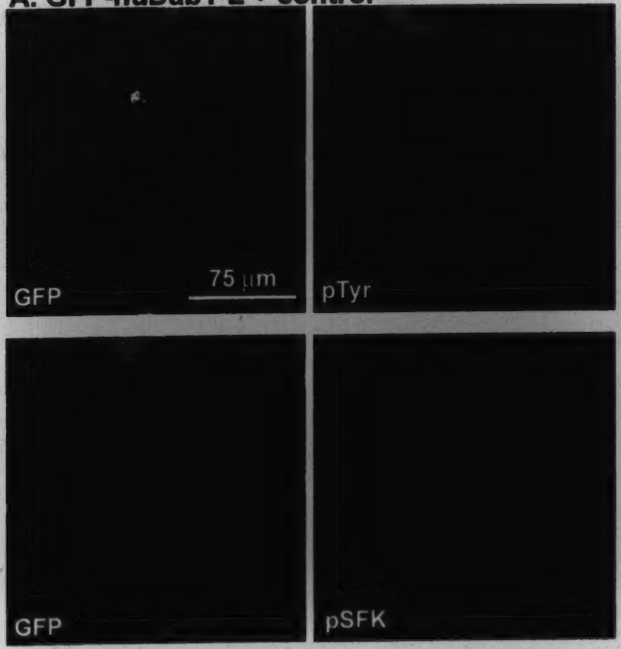
activation and neurite formation. To verify that tyrosine-198 itself is phosphorylated in the transfectants and to determine whether other tyrosine residues might mediate the observed changes in SFK activity and cellular morphology, Dab1 Y→F double and triple mutants were analyzed (Fig. 1). As expected, cells expressing mutants that included the Y198F substitution (Dab1$^{Y185F/Y198F}$, Dab1$^{Y198F/Y220F}$, Dab1$^{Y198F/Y232F}$, Dab1$^{Y185F/Y198F/Y220F}$, Dab1$^{Y185F/Y198F/Y232F}$, Dab1$^{Y198F/Y220F/Y232F}$) (Figs. 4.1F, 4.1I, 4.1J, 4.1L, 4.1M, 4.1O) displayed identical morphology and properties as those expressing the Dab1^{Y198F} single mutant. In agreement with results obtained with Dab1^{Y232F}, cells expressing Y232F-containing double mutants (Dab1$^{Y185F/Y232F}$and Dab1$^{Y220F/Y232F}$) or the Dab1$^{Y185F/Y220F/Y232F}$ triple-mutant had elevated levels of phosphotyrosine but no evidence of SFK activation (Figs. 4.1H, 4.1K, 4.1N). However, these double and triple mutants had a morphology that was highly reminiscent of Dab1-E-expressing cells. Interestingly, cells expressing the Dab1$^{Y185F/Y220F}$ double-mutant (Fig. 4.1G) showed a phenotype similar to that of Dab1^{Y232F}-expressing cells; ie. intermediate morphology, high phosphotyrosine levels and reduced activation of SFKs. Combined, these data indicate that while tyrosine-198 is a primary and essential residue required for Reelin-mediated Dab1 tyrosine phosphorylation, tyrosine-232, combined with either tyrosine-185 or tyrosine-220, are required for full Dab1^{Y198}-mediated activation of SFKs and neurite formation.

4.3.2 SHP-2 suppresses Dab1-mediated SFK activation and neurite formation

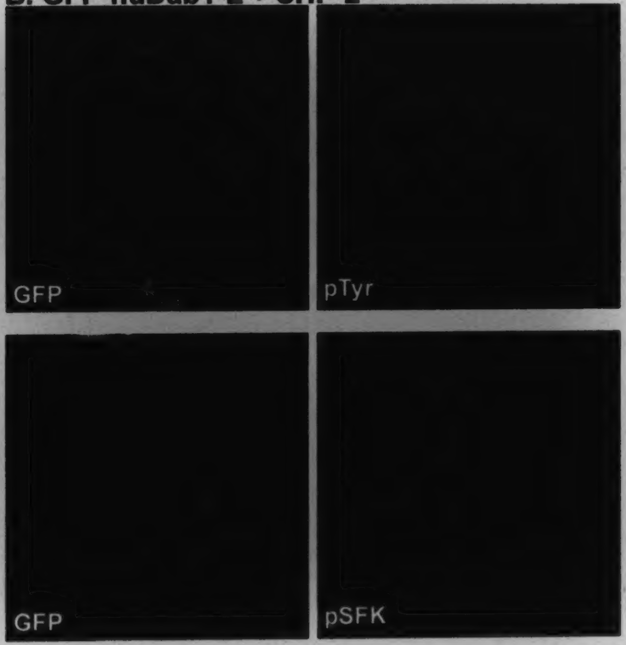
Tyrosine phosphorylated Dab1 has recently been shown to interact with the protein tyrosine phosphatase SHP-2 (Ballif *et al.*, 2004). Analysis of Dab1 tyrosine phosphorylation sites shows that both tyrosine-185 (A-V-pY^{185}-Q-T-I) and tyrosine-198 (P-V-pY^{198}-Q-Y-I) resemble the consensus SHP-2 binding motif, [I/V/L]-x-(p)Y-x-x-[I/V/L] (Ravetch and Lanier, 2000). As Dab1 is predominantly phosphorylated at tyrosine-198 and not tyrosine-185 (Keshvara *et al.*, 2001; Katyal and Godbout, 2004), it is likely that the phosphoDab1-SHP-2 interaction occurs via tyrosine-198. To determine whether SHP-2 might play a role in Dab1 tyrosine phosphorylation and SFK activation, we transfected primary chick retinal cultures with GFP-huDab1-L and SHP-2 expression constructs. Consistent with previous results, cells co-transfected with pEFGP-huDab1-L and a vector control (pRK5) showed similar morphology and properties to those of cells expressing Dab1-L alone (ie. process formation, elevated levels of phosphotyrosine and SFK activation) (Fig. 4.2A) (Katyal and Godbout, 2004). However, the morphology of cells co-transfected with GFP-huDab1-L and a SHP-2 expression construct (pRK5-SHP-2) was reminiscent of huDab1-E expressing retinal cells (Fig. 4.2B). Immunofluorescence staining with anti-phosphotyrosine and anti-phosphoSFK^{Y416} antibodies yielded surprising results. In contrast to cells transfected with huDab1-E alone, tyrosine phosphorylation was still detected in Dab1-L/SHP-2 co-transfected cells, with the signal primarily localized to the cell membrane. However, SFK was not activated in these co-transfected

Fig. 4.2 - Analysis of primary chick retinal cells co-transfected with human Dab1-L and either pRK5 vector (**A**) or pRK5-SHP-2 (**B**). GFP-Dab1 expressing cells (shown in green) were fixed and stained with mouse anti-phosphotyrosine or mouse anti-phosphoSFK^{416} antibodies, followed by goat-anti mouse Alex 555-conjugated secondary antibody (shown in red).

A. GFP-huDab1-L + control


GFP
75 μm
pTyr
GFP
pSFK

B. GFP-huDab1-L + SHP-2


GFP
pTyr
GFP
pSFK

cells. These data indicate that SHP-2 does not dephosphorylate Dab1, yet it somehow precludes phosphoDab1-mediated activation of SFKs.

4.3.3 Identification of additional putative Dab1-interacting proteins

A number of investigations have used a variety of techniques to identify Dab1-interacting proteins. Some of these interactions are shown in Chapter 1, Fig 1.11. In order to identify proteins that interact with Dab1-E or Dab1-L, we performed a yeast two-hybrid screen using a human fetal brain cDNA library. Two human Dab1 baits were used: Dab1-E, from aa 1-257, and Dab1-L, from aa 1-273. Thus, the Dab1-L bait contains all four tyrosine phosphorylation sites while Dab1-E lacks Y185 and Y198 (two SFK recognition sites) but retains Y220 and Y232 (two Abl kinase/Crk recognition sites) thereby allowing for detection of interactions specific to these regions. Both constructs include the Dab1 N-terminus, a region previously shown to bind a number of proteins such as ApoER2, VLDLR, APP, APLP1, APLP2, etc. (as described in Chapter 1). The Dab1-E bait also contains a unique region encoding 17 amino acids with no recognizable motifs (as described in Chapter 3). Multiple baits spanning larger (full-length) and smaller (tyrosine phosphorylation sites, Dab1-E-specific 17 amino acid domain) regions were generated; however, their transformation into AH109 yeast cells resulted in premature activation (leaky expression) of the HIS3 nutritional reporter, thereby precluding their effective use for GAL4-based yeast two-hybrid analysis. For both screens, ~2.5 X 10^6 independent clones were analyzed. The Dab1-E screen yielded 20 HIS3/ADE2/lacZ-positive colonies and plasmid DNA was successfully isolated and sequenced from 15 colonies. The Dab1-L screen yielded 29 such colonies and plasmid DNA was isolated and sequenced from 24 colonies. The identities of the two-hybrid positive clones analyzed to date are summarized in Table 4.1. As an indication of the success of our screen, amyloid precursor-like protein 1 (APLP1) and APLP2, were identified in 6 of 15 Dab1-E-derived positives and 10 of 24 Dab1-L-derived positives. Both APLP1 and APLP2 have previously been shown to interact with the Dab1 N-terminus (Homayouni *et al.*, 1999).

Notable putative interacting proteins identified in the Dab1-E screen include: alpha C-adaptin, which contains the adaptor protein-2 (AP-2) alpha subunit ear domain involved in clathrin-mediated endocytosis and endocytic trafficking (Morris and Cooper, 2001); kinesin 3B (KIF3B), implicated in membrane organelle transport and neural development (Yamazaki *et al.*, 1995; Nonaka *et al.*, 1998); ephrin A3, implicated in CNS axon arborization and pathfinding (Gao *et al.*, 1999); and protein tyrosine phosphatase type IV-A2 (PRL-2). Interacting proteins identified using Dab1-L as the bait include: 5-azacytidine induced protein-1, a protein with homology to myosin motor proteins; neuronal F-box protein 42 (NFB42), a component of the neuronal specific Skp1-Cullin1-NFB42-Rbx1 [SCF(NFB42)] E3 ubiquitin ligase (Murai-Takebe *et al.*, 2004) and SGT1, a protein also shown to associate with the SCF E3-ubiquitin ligase complex (Kitagawa *et al.*, 1999).

To determine whether any of these putative interactors were specific to Dab1-E or Dab1-L, prey plasmids were transformed into Dab1-E and Dab-L-

Dab1-E Two-hybrid Screen	**Dab1-L Two-hybrid Screen**
Total number of HIS3/ADE2/LacZ positive colonies = 20	*Total number of HIS3/ADE2/LacZ positive colonies = 25*
Sequence identity of positives (# of positives)	*Sequence identity of positives (# of positives)*
APLP1, Amyloid precursor protein like-1 (6)	**APLP1**, Amyloid precursor protein like-1 (9)
alphaC-adaptin, adaptor-protein 2-alpha subunit (1)	**APLP2**, Amyloid precursor protein like-2 (1)
KIF3B, kinesin II subunit (1)	**NFB42/FBOX2**, neuronal F-box protein 42 (1)
Ephrin-A3, ligand for Eph receptor (1)	**SGT1**, suppressor of G2 allele oc SKP1 (2)
PRL-2, protein tyrosine phosphatase type IV-A2 (1)	**PIAS3**, protein inhibitor of activated STAT3 (1)
SUV420H, Suppressor of variegation 420 homologue (2)	**Azi1**, 5-azacytidine-induced gene-1 (1)
PSID (2)	**TTC-1**, tetratricopeptide-1 (1)
ESTs/unknown proteins (1)	ESTs/unknown proteins (9)
not sequenced (5)	

Table 4.1 - Identity of candiate Dab1-interacting proteins isolated by yeast two-hybrid analysis of Dab1-E and Dab-L

Clones derived from the Dab1-L library screen were isolated and sequenced by Tina Gao, a graduate student in the lab expressing AH109 cells using the small scale transformation protocol and grown on SD/-trp/-leu/-his/-ade media for 5 days. Each prey plasmid, whether co-transformed with Dab1-E or Dab1-L, grew equally well, indicating that none of the peptides encoded by the positive clones had any particular preference for either Dab1 isoform. These results suggest that the putative Dab1-interacting proteins identified in our screen do not require the regions encompassing Y185 and Y198 or the Dab1-E-specific 17 aa insertion domain for their interaction with Dab1.

4.4 DISCUSSION

4.4.1 Dab1 tyrosine mutation analysis

In this study, we attempt to dissect the molecular mechanism by which tyrosine phosphorylation regulates Dab1-mediated SFK activation and neuritogenesis. Through our mutation analysis, we have found that Dab1 tyrosine phosphorylation is hierarchical in nature, with tyrosine-198 being the primary Reelin-induced phosphorylation site that is essential for SFK activation and formation of processes. These results are consistent with reports in the literature including our own (Keshvara *et al.*, 2001; Katyal and Godbout, 2004). The novelty in our findings resides in the data derived from our combinatorial Y→F mutation analysis. In addition to tyrosine-198, our data reveal a requirement for additional Dab1 tyrosine residues in SFK-activation and process formation with tyrosine-232, and either one of tyrosine-185 or tyrosine-220, being essential for full Dab1 activity. Based on our data, tyrosine-185 and tyrosine-220 can compensate for each other, a surprising finding considering that tyrosine-185

(YQTI) and tyrosine-220 (YQVP) differ in their sequence motifs. Our results suggest a compensatory mechanism for tyrosine-185 and tyrosine-220-mediated signaling, through activation of different cellular pathways. Whether tyrosines 185, 200 and 232 are phosphorylated subsequent to phosphorylation of tyrosine-198 and activation of SFKs will require further analysis. For example, phosphotyrosines 185, 220 and 232-specific antibodies in conjunction with expression of a phosphotyrosine-mimetic Dab1 Tyr198Glu mutant would address the phosphorylation status of all four tyrosines (Keshvara *et al.*, 2001; Potter *et al.*, 2005). Furthermore, transgenic mice expressing different single, double and triple Y→F substitutions (all involving Y198F) in a *Dab1*$^{-/-}$ background would provide greater insight into how individual tyrosine residues contribute to Reelin-Dab1 signaling. A biochemical analysis of single, double, triple and quadruple Dab1 Y→F mutants has recently been described by Ballif and colleagues (2004). In their study, non-neuronal HEK293T cells were co-transfected with an expression construct for activated Fyn kinase and different combinations of Dab1 tyrosine mutants. This system was used to identify key phosphotyrosine sites required for Dab1 interaction with the Crk family of adaptor proteins. Dab1-Crk interaction was found to be dependent on phosphorylation of both tyrosine-220 and tyrosine-232. Our study is novel in that it adds to other work in this area by providing an effective biological assay to examine how alteration of Dab1 tyrosine residues affects endogenous biochemical pathways and neuronal cell morphology. Therefore, our study gives biologically relevance to the function of

Dab1 tyrosine phosphorylation in Reelin-mediated neuronal cell migration and/or process formation.

4.4.2 Role of SHP-2 in Dab1 signaling

Ballif *et al.* (2004) originally identified SHP-2 as a Dab1-interacting protein through Dab1 phosphopeptide affinity purification. However, the authors were unable to co-immunoprecipitate Dab1 with SHP-2 from embryonic cortical extracts, perhaps because Dab1 was dephosphorylated in their experiments as tyrosine phosphomodifications are known to be labile (Burke, Jr. and Lee, 2003). Furthermore, not all phosphotyrosine-dependent associations can be detected through biochemical assays, especially those involving a protein tyrosine phosphatase such as SHP-2. Using our primary retinal cultures, we have identified a possible function for SHP-2 in the regulation of Reelin-Dab1 signaling. Although Dab1 remains tyrosine phosphorylated in the presence of SHP-2, the immunoreactive pattern is altered, with immunostaining primarily found near the plasma membrane. Furthermore, we have clear evidence indicating that SHP-2 can suppress Dab1-mediated SFK activation and the associated morphological changes. We propose two mechanisms to explain how SHP-2 might be involved in Reelin-Dab1 signaling. Under the first mechanism, Reelin-mediated Dab1 tyrosine phosphorylation induces SFK phosphoactivation which, in turn, activates SHP-2. Activated SHP-2 phosphatase would then dephosphorylate and inactivate SFKs. This mechanism implies the existence of a SFK regulatory loop in which Dab1 and SHP-2 function to fine-tune SFK activity in Reelin-stimulated neurons. Whether or not SFKs and SHP-2 are functionally interrelated remains controversial (Neel *et al.*, 2003). Fyn tyrosine kinase has been shown to bind and activate SHP-2 (Tang *et al.*, 1999). Conversely, SHP-2 has been found to signal upstream of SFKs and regulate the inhibitory Src tyr-527 phosphorylation event (Cunnick *et al.*, 2002). In light of this, the first mechanism appears unlikely as we do not observe any phosphoSFK^{416} immunoreactivity in GFP-Dab1-L/SHP-2 co-transfected cells to account for a population of activated SFK molecules that have yet to undergo SHP-2-mediated dephosphorylation. A second, and perhaps more likely mechanism would involve the recruitment of SHP-2 to Dab1 upon Reelin-mediated Dab1 phosphorylation at tyrosine-198, thus sterically preventing Dab1-SFK interaction. As a consequence, Dab1-mediated SFK activation would be inhibited. Like most phosphoDab1-interacting proteins, SHP-2 contains an SH2 domain. SHP-2 has previously been shown to bind other tyrosine phosphorylated scaffolding adaptor proteins through its SH2 domain and functions as both a positive and negative regulator of tyrosine kinase signaling through phosphatase-independent mechanisms (Pawson and Scott, 1997; Hof *et al.*, 1998). It is possible that Dab1 phosphorylation at tyrosine-198 may serve as a regulatory branchpoint, to either recruit and activate SFKs and accompanying signaling events, or to bind and induce SHP-2 and accompanying signaling events thereby preventing SFK activation.

4.4.3 Identification of additional Dab1-interacting proteins

Our two-hybrid analysis, thus far, has failed to identify any Dab1 isoform/tyrosine-specific interactions which was the initial goal of our study. Future two-hybrid analysis may require the phosphorylation of Dab1 tyrosine residues through the use of SFK-expressing yeast strains. Using yeast integration vectors, expression cassettes containing cDNAs to constitutively active Src or Fyn tyrosine kinases can be integrated into the yeast genome. This strategy was employed in the identification of Nckβ as a phosphotyrosine-dependent Dab1-interacting protein (Pramatarova *et al.*, 2003). These specialized yeast strains could also be used to screen Dab1 mutants harboring various combinations of Y→F substitutions to identify site-specific Dab1 phosphotyrosine-protein interactions. An alternative strategy would be to screen Dab1 bait proteins harboring specific Y→E substitutions thereby producing phosphotyrosine mimetic sites.

Despite our failure to identify tyrosine-specific interactions, our two-hybrid screen was successful in that it yielded a number of interesting putative Dab1-interacting proteins. For example, our discovery that Dab1 interacts with alphaC-adaptin may provide the missing link needed to explain how Reelin/VLDLR are internalized into clathrin-coated vesicles and how they undergo intracellular trafficking (D'Arcangelo *et al.*, 1999; Morimura *et al.*, 2005). Previous studies have shown that the second member of the Dab1 protein family, Dab2, forms a tripartite complex with the myosin VI motor protein and AP-2 alpha subunit ear domain (alphaC-adaptin) which allows clathrin-mediated endocytosis of FxNPxY motif-containing receptors such as the LDLR receptor family (Morris and Cooper, 2001; Morris *et al.*, 2002a; Morris *et al.*, 2002b). The C-terminal ear domain of alphaC-adaptin associates with two aspartate(D)-proline(P)-phenylalanine(F) motifs located between the central region and the myosin VI binding domain in Dab2. Mutation of these motifs abrogates the Dab2-AP-2 alpha subunit interaction and prevents receptor internalization. The cDNA fragment recovered in our Dab1 two-hybrid screen encodes only the alphaC-adaptin ear domain, thereby suggesting a conserved Dab1/Dab2 interaction motif within alphaC-adaptin. Although Dab1 contains a D-P-F motif at the C-terminus (aa 517-519 in Dab1-E and 537-537 in Dab-L), our two-hybrid bait does not include this region. This suggests that an alternative motif within Dab1 mediates its interaction with alphaC-adaptin. AP-2 interactions with its substrates are known to occur through other tripeptide motifs. In Dab1, these may include (with corresponding aa in Dab1-E/Dab1-L indicated): $D^{157/157}$- L-F, $E^{229/247}$-L-F and $D^{251/269}$- A-F which are all found in our Dab1 baits.

Several lines of evidence suggest the involvement of Dab1 in clathrin-mediated endocytosis. First, fluorescent immunolocalization of Dab1 in Reelin-stimulated neurons shows a similar clathrin-like punctuate staining pattern as seen upon Dab2 immunostaining (D'Arcangelo *et al.*, 1999). Second, like Dab2, the Dab1 C-terminus interacts with myosin VI (Morris *et al.*, 2002a). Third, Dab1 colocalizes with internalized Reelin and levels of Reelin vesicle internalization are 60% higher in wild-type cortical neurons compared to Dab1-deficient (*yotari*) neurons (Morimura *et al.*, 2005).

In addition to alphaC-adaptin, our two-hybrid screen revealed novel interactions with two cytoskeletal motor proteins: 5-azacytidine induced gene-1 and KIF3B. Although the function of 5-azacytidine induced gene-1 remains unknown, this gene shows strong homology to a variety of actin-associated myosin motor proteins. As mentioned above, Dab1 is known to associate with myosin VI (Morris *et al.*, 2002a). Our finding of another Dab1-myosin protein interaction implicates Dab1 in alternative actin-mediated transport pathways in addition to myosin VI/clathrin-mediated endocytic trafficking. KIF3B is a subunit of the microtubule motor protein, kinesin II, which is involved in microtubule-mediated membrane organelle transport and neural development (Yamazaki *et al.*, 1995; Nonaka *et al.*, 1998). Recently, Dab1 has been found to associate with LIS1, a dynein/dynactin-associated protein also implicated in microtubule-mediated transport (Assadi *et al.*, 2003). Both dynein/dynactin (minus-end transport) and kinesin (plus-end transport) have been shown to undergo a coordinated "tug-of-war" in fine-tuning intracellular transport of macromolecular cargo along microtubules (Gross, 2003). The association of Dab1 with LIS1 and KIF3B suggests an intriguing mechanism by which Dab1 may regulate both forms of directed transport. This would imply an additional role for Reelin-Dab1-signaling in regulating the bidirectionality of microtubule motor proteins in order to specify the final destination of intracellular cargo.

Dab1 has previously been shown to undergo Reelin-induced SFK-dependent ubiquination and proteolytic degradation via the Cbl pathway (Arnaud *et al.*, 2003a; Bock *et al.*, 2004; Suetsugu *et al.*, 2004). Cbl is an SH2-containing

E3-ubiquitin ligase and allosterically activates an E2-ubiquitin conjugating enzyme through its RING finger domain (Joazeiro *et al.*, 1999). Recently, Dab1 has also been shown to associate with and inhibit the E3-ubiquitin ligase SIAH-1A. Our identification of NFB42 and SGT1 as Dab1-interacting proteins further implicates Dab1 in the regulation of ubiquination. NFB42 is part of the neuronal specific Skp1-Cullin1-NFB42-Rbx1 (SCF(NFB42)) E3 ubiquitin ligase complex which associates with both SIAH-1 and SGT1 to promote degradation of β-catenin (Matsuzawa and Reed, 2001; Murai-Takebe *et al.*, 2004). Although we do not know the functional significance of the Dab1-NFB42/SGT1 interactions, it is possible that Dab1 may regulate their ubiquitin ligation activities in a similar manner as with SIAH-1A.

In summary, this analysis sheds new light on how individual tyrosine phosphorylation sites regulate SFK activation and neuritogenesis. We have found that combinatorial phosphorylation involving tyrosine-198 and at least one other tyrosine site is required to mediate Reelin-Dab1-associated phenotypes in our retinal cultures. We have also identified a function for the SHP-2 phosphatase in regulating Reelin-Dab1 signaling. Furthermore, we have identified a number of novel Dab1-interacting proteins which suggest additional intracellular functions for Dab1 in cytoskeletal dynamics, neuronal cell migration and protein degradation.

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Chapter 5

Discussion and Future Directions

5.1 DISCUSSION

5.1.1 Summary of results

We have identified a new alternatively-spliced form of Dab1, Dab1-E, from the developing chick retina and brain (Chapter 2). In the retina, Dab1-E is found in retinal progenitor cells. A unique feature of Dab1-E is the loss of two SFK recognition motifs (encompassed within exons 7 and 8) including Reelin-responsive Y198, while two Abl/Crk recognition motifs are retained (Songyang *et al.*, 1993; Howell *et al.*, 1997a; Howell *et al.*, 2000; Keshvara *et al.*, 2001). In addition, we have found that alternative exon 9B is included in Dab1-E, perhaps providing a function unique to Dab1-E. Expression of Dab1-E in our primary chick retinal cultures results in these cells adopting a round undifferentiated cell morphology. These cells show a lack of Dab1 tyrosine phosphorylation and absence of SFK activation.

There is a switch from Dab1-E to Dab1-L when retinal cells begin to undergo migration and differentiation (at ED5 - ED7). Dab1-L corresponds to the previously described human and murine (Dab555) form of Dab1 characterized by inclusion of the two exons encompassing the two SFK recognition motifs and exclusion of alternative exon 9B. Our *in situ* hybridization analyses indicate that this splice form is found in two retinal cell types, amacrine and ganglion. Furthermore, the inner plexiform layer, a synaptic layer interconnecting amacrine cells with the ganglion cell layer, is also positive for Dab1-L expression. Based on retinal immunofluorescence and primary chick retina transfection data, we find that Dab1-L expression increases Dab1 tyrosine phosphorylation, SFK activation and induces formation of elongated neurite-like processes. These Dab1-L-dependent morphological and biochemical properties are Reelin-dependent as inhibition of Reelin or loss of Reelin-mediated Dab1 phosphorylation at Y198 (using a Y198F mutant) results in Dab1-E-like properties (ie. reduced phosphotyrosine levels, no SFK activation and round undifferentiated morphology).

We have also analyzed the expression of Dab1-E and Dab1-L in human fetal retina (Chapter 3). Immunofluorescent labeling of human Dab1 protein in fetal retina tissue sections combined with huDab1 transfection analysis of our primary chick retina cultures suggest that the functions associated with the human Dab1 isoforms are similar to those of chicken Dab1 isoforms. These data demonstrate an evolutionarily conserved role for Dab1-E, Dab1-L and Dab1 isoform switching in vertebrate neuronal development. We also show that Dab1 alternative splicing is deregulated in two tumours of neuronal cell origin, retinoblastoma and neuroblastoma and that Dab1 and Reelin proteins are not expressed in these tumours. Based on these data, we propose that inactivation of Reelin-Dab1 signaling is required for tumorigenesis, reflecting the proliferative and undifferentiated properties of these tumors.

Through mutation analysis of Dab1-L, we have also deciphered the contribution of each tyrosine (Y185, Y198, Y220 and Y232) to Dab1 activity (Chapter 4). We show that Y198 is primarily required for Dab1 tyrosine phosphorylation and SFK activation, while Y232, combined with Y220 or Y185, are required for SFK activation. These data indicate the hierarchical nature of these tyrosines with regards to Dab1 function with Y198>Y232>Y220/Y185. Furthermore, these data also indicate the context by which exclusion of Y198 in Dab1-E results in loss of SFK activation despite the retention of Y220 and Y232.

5.1.2 Developmental model for Dab1 isoform switching

Our studies point to a novel mechanism in the regulation of Reelin signaling (Fig. 5.1). We propose that Dab1-E expression early in neuronal development represents a flexible way for immature neurons to prevent premature Reelin signaling through decoupling of the Reelin-Dab1 signaling pathway. By splicing out Y198 and precluding Reelin-mediated Dab1 tyrosine phosphorylation, SFKs are no longer Reelin-responsive and downstream Reelin/SFK-dependent cytoskeletal events are blocked (Fig. 5.1A). Restriction of Dab1-E expression to retinal progenitor cells lends support to a role for this early isoform in the suppression of premature neuronal differentiation. Furthermore, our finding that Dab1 and Reelin protein expression are lost in retinoblastoma and neuroblastoma tumour cell lines provides further evidence for a requirement to decouple this pathway in proliferating cells. When Dab1-expressing cells are ready to undergo Reelin-mediated migration and/or neuronal differentiation, we hypothesize that specific developmental signals modulate recognition of exons 7, 8 and 9B splice donor/acceptor sequences of the *Dab1* pre-mRNA, thereby allowing the switch to Reelin-responsive *Dab1-L*. This switch acts to recouple Reelin-stimulated cell signaling resulting in SFK activation and Reelin-mediated modulation of the cell cytoskeleton resulting in migration and neuritogenesis (Fig.

5.1A). Such a mechanism would allow a heterogeneous population of cells, expressing either Dab1-E or Dab1-L, to share a common Reelin-enriched environment while undergoing divergent biochemical functions (Fig. 5.1B). During retinal and brain development, such a mechanism might be highly conducive to cell lamination. Our RT-PCR data indicate that Dab1-E to Dab1-L switching is asynchronous as cDNAs corresponding to both splice forms are amplified at intermediate stages of retinal development in chick (ED7 and ED10) and in human (8 wks gestation) as well as in brain development (ED3.5 and ED5 in chick and 8 wks gestation in human). Therefore, we further hypothesize that asynchronous Dab1 isoform switching contributes to the specification of particular neuronal cell layers and formation of laminated structures required for proper retinal and brain development (Fig. 5.1B). Dab1 isoform switching at early developmental stages may destine Dab1-L-expressing cells to differentiate into neuronal cell lineages that appear earlier such as ganglion cells, while switching at later developmental stages may mark Dab1-L-expressing cells for differentiation along lineages that appear later such as amacrine cells. In support of this hypothesis, we find that at ED5 when 80% of cells are proliferating (Dutting *et al.*, 1983), Dab1-E is initially expressed throughout the retina (cells which have not undergone commitment to a particular cell lineage). However, at later stages of differentiation, only two cell types, amacrine and ganglion, express Dab1-L. Furthermore, while specification of ganglion cells occurs earlier than that of amacrine cells, ganglion and amacrine cells are found in adjacent layers, suggesting a close relationship in the time of appearance of these two cell types.

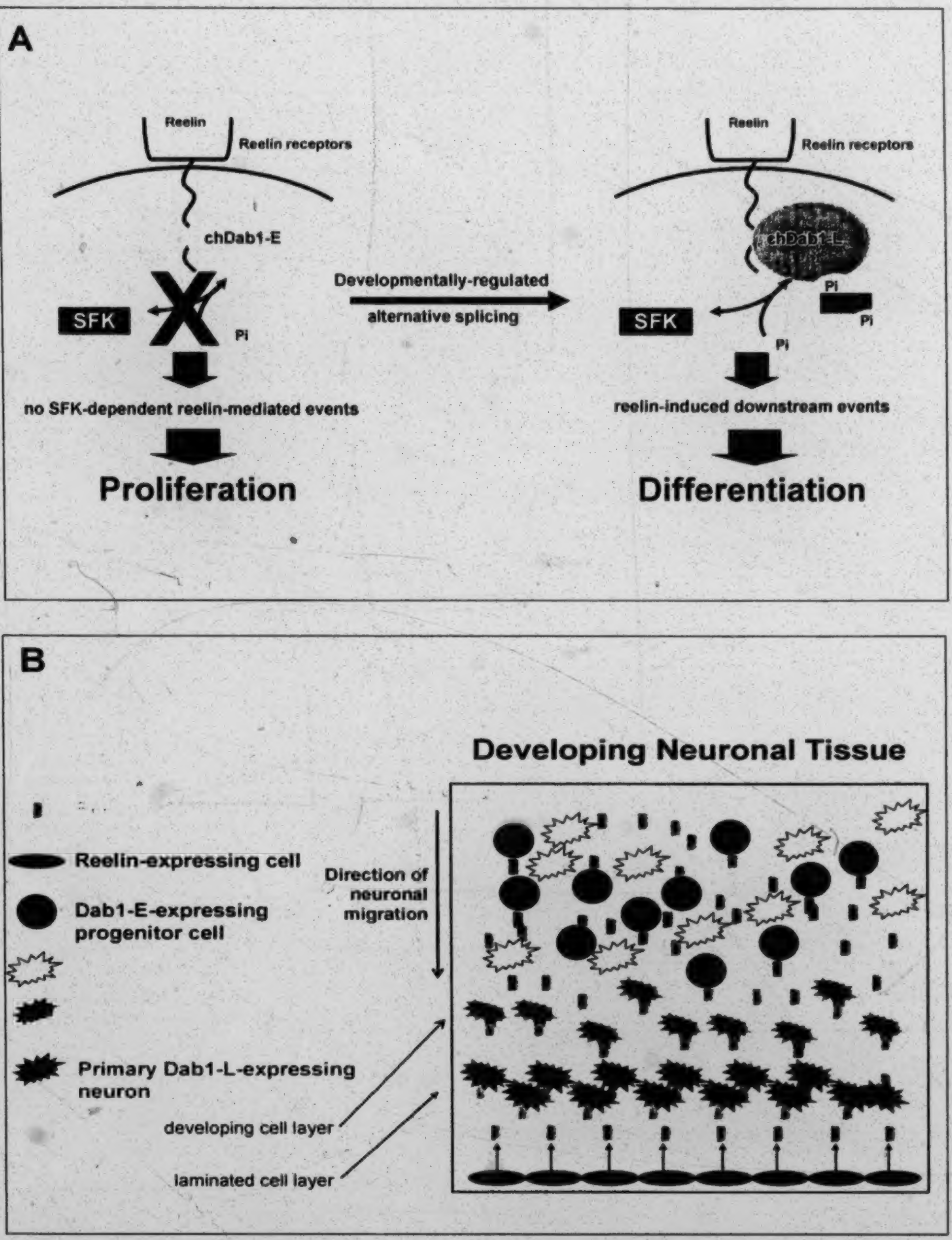
A
Reelin
Reelin receptors
chDab1-E
SFK
Pi
no SFK-dependent reelin-mediated events
Proliferation
Developmentally-regulated
alternative splicing
Reelin
Reelin receptors
chDab1-L
Pi
Pi
SFK
Pi
reelin-induced downstream events
Differentiation
B
Developing Neuronal Tissue
Reelin-expressing cell
Dab1-E-expressing progenitor cell
Primary Dab1-L-expressing neuron
Direction of neuronal migration
developing cell layer
laminated cell layer

Fig. 5.1 - Model for Reelin-Dab1 signaling in the developing chick retina. **(A)** Reelin binding to its receptors activates Dab1-L, resulting in activation of SFKs and downstream effectors, leading to the formation of processes associated with cell differentiation. The absence of SFK phosphorylation sites in Dab1-E prevents this signaling cascade. **(B)** Proposed model for asynchronous uncoupling-coupling of the Reelin-Dab1 signaling cascade depending on whether retinal cells express Dab1-E or Dab1-L. As sub-populations of cells switch expression from Dab1-E to Dab1-L, they will migrate towards Reelin-producing cells where they will position themselves into cell layers.

5.1.3 Parallels in Reelin/Dab1-mediated cell lamination of the inner retina and brain

In the brain, Cajal-Retzius (CR) neurons secrete Reelin protein which, in turn, binds to Dab1-expressing pyramidal or cortical migratory neurons located at the ventricular zone (Tissir and Goffinet, 2003). Reelin binding to these cortical neurons directs their migration towards CR neurons resulting in their organization into distinct cell layers. In the retina, we and other groups have found Reelin to be expressed in ganglion cells (discussed in the next paragraph) (Bernier *et al.*, 2000; Rice *et al.*, 2001; our unpublished data, 2005). Furthermore, Dab1-L-expressing ganglion and amacrine cells are derived from the Dab1-E-expressing (Reelin-unresponsive) retinal progenitor cell pool at the ventricular zone. We suggest that retinal ganglion cells may function analogously to cortical CR cells while amacrine cells may function analogously to pyramidal neurons. Like pyramidal neurons, we propose that Dab1-L-expressing amacrine cells undergo directional migration towards Reelin-secreting retinal ganglion cells (located at the inner aspect of the retina). The remaining neuronal cell types that are derived from the retinal ventricular zone (photoreceptor, horizontal, bipolar and interplexiform) no longer express Dab1. These cells either migrate towards the outer aspect of the retina (photoreceptor and horizontal) or remain relatively static (bipolar and interplexiform). Therefore, we suggest that, in addition to lamination of the inner retina, Reelin-Dab1 signaling may specify the direction of cell migration during retinal development.

It is important to note that both CR neurons and ganglion cells co-express Reelin and Dab1 proteins. In CR cells, this co-expression is believed to reflect a need for Reelin and Dab1 in both CR development and CR function after neuronal maturation (Deguchi *et al.*, 2003). In the retina, ganglion cell dendrites must form connections with amacrine and bipolar cells (in the IPL) while ganglion cell axons must extend through the optic nerve and connect with dorsal lateral geniculate nucleus (LGN) cells in the brain (Guido and Lu, 1995). These connections allow the processed visual signal to be transduced from the INL to the GCL where the signal is projected through LGN cells to the visual cortex. Interestingly, LGN cells express high levels of Reelin protein in chick and mouse (Schiffmann *et al.*, 1997; Bernier *et al.*, 2000). These data suggest that retinal ganglion cells may have dual roles with respect to Reelin-Dab1 signaling. First, as postulated above, secretion of Reelin protein by ganglion cells may specify amacrine cell positioning, layering and formation of interneuronal connections at the IPL. Second, Reelin secreted by LGN cells may bind Dab1-expressing ganglion cells, regulate GCL axonal outgrowth through the optic nerve and specify the formation of GCL-LGN connections at the visual cortex. A third role may be that ganglion cells can autoregulate Reelin-Dab1 signaling in a cell-autonomous manner. As ganglion cells express both Reelin and Dab1, secretion and subsequent binding of Reelin may be a way for retinal ganglion cells to regulate their own Reelin-dependent migration and neuritogenesis.

5.1.4 Evidence supporting a functional role for Dab1-E

In defining a role for Dab1-E in decoupling the Reelin-Dab1 signaling pathway, one may ask whether a need exists for expression of Dab1-E. One might speculate that it would be more advantageous for cells to simply not express Dab1-E as it would conserve valuable cellular resources with no consequence to Reelin signaling. However, examination of the specific inclusion and exclusion of key regions arising from Dab1 alternative splicing and formation of the *Dab1-E* splice form suggests a requirement for cellular expression of Dab1-E. First, our RT-PCR results demonstrate the inclusion of alternative exon 9B only in Dab1-E and not in Dab1-L. This domain does not correspond to any known motif published in the literature. However, *in situ* hybridization studies combined with RT-PCR analysis of embryonic chick and mouse tissue indicate that Dab1-E (in chick) and exon 9B (in mouse) are abundantly expressed in proliferative neural progenitor cells suggesting a functional need for Dab1-E (Bar *et al.*, 2003; Katyal and Godbout, 2004). Likewise, the developmentally-regulated exclusion of exon 9B from Dab1 suggests that Dab1-E expression is not compatible with Dab1-L expression during neuronal differentiation. Based on these data, we postulate that Dab1-E expression may have inhibitory effects on Dab1-L activity.

Second, the specific nature of alternative splicing that gives rise to Dab1-E suggest a specific requirement for Abl/Crk recognition motifs in Dab1 function early in neuronal development. In addition to the loss of the Y^{198}-SFK motif, the loss of exons 7 and 8 and the resulting splicing of exon 6 to exon 9 "regenerates" the Abl/Crk motif (Y-Q-V-P) through conversion of the Y^{185}-SFK motif to a Y^{220}-

like Abl/Crk motif. Thus, Dab1-E alternative splicing ensures the presence of two Abl/Crk motifs, suggesting an important role associated with Dab1-E function in neural progenitor cells. Interestingly, Dab1 Y220 and Y232 bind the Crk family of adaptor proteins (CrkI, CrkII, CrkL, Nckα and Nckβ) suggesting roles for Dab1-E specifically involving this family of proteins (Howell *et al.*, 1997a; Pramatarova *et al.*, 2003; Ballif *et al.*, 2004; Chen *et al.*, 2004; Huang *et al.*, 2004).

Third, although our yeast two-hybrid screen failed to identify isoform-specific interacting proteins, we and other groups have identified numerous Dab1-interacting proteins that are involved in the cell cycle (SGT1), ubiquination (NFB42), modulation of protein activity (PIAS3), intracellular transport and trafficking (KIF3B, Azi1, alphaC-adaptin and myosin VI) and actin polymerization (N-WASP) (Morris *et al.*, 2002; Suetsugu *et al.*, 2004; our unpublished data, 2005). As these interactions can occur with either form of Dab1 and are independent of Dab1 SFK motifs, they implicate Dab1 in pathways independent of Reelin.

A fourth reason is derived from a re-evaluation of the phenotypes exhibited by mouse mutants. Previous work has shown that mice deficient in *Reelin*, *Dab1* or double-deficient in *ApoER2* and *VLDLR* show similar neuronal positioning defects (D'Arcangelo *et al.*, 1995; Sheldon *et al.*, 1997; Howell *et al.*, 1997b; D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Trommsdorff *et al.*, 1999). However, a closer examination of these mutant mice reveals differences in neuronal layering and gross brain morphology. For example, re-examination of the original data presented in Yoneshima *et al.* (1997) reveals differences between *reeler* (*Reelin*$^{-/-}$) and *yotari* (*Dab1*$^{-/-}$) cerebella. These include a larger cerebellum in *reeler* mice compared to *yotari* mice (Chapter 1, Fig. 1.8), and a difference in the scattering patterns of Purkinje cells (Yoneshima *et al.*, 1997). The phenotypic similarities in these mice reflect the fact that both Reelin and Dab1 are found in the same pathway; however, the differences imply a degree of functional independence.

5.1.5 Dab1 transcription and RNA processing are tightly regulated during retinal maturation

The regulation of Dab1 expression and Dab1 isoform switching in specific retinal cell types suggests a sophisticated, developmentally-regulated mechanism controlling both Dab1 transcription and *Dab1* alternative splicing. In the vertebrate retina, we find that Dab1 is first expressed in retinal progenitor cells. In these cells, the Dab1-E splice form is the primary Dab1 species that is expressed. Upon retinal differentiation, two events occur which suggest developmental- and cell-specific regulation of *Dab1* transcription and RNA processing. First, as retinal development progresses, Dab1 RNA and protein become restricted to amacrine and ganglion cells and are lost in all other mature cell types. This would indicate the existence of a cell type-specific transcriptional repression mechanism (with respect to Dab1 expression) in non-amacrine/ganglion cells. As all differentiated cell types are derived from one type of precursor (Turner and Cepko, 1987), this mechanism would function to actively repress *Dab1* transcription in specific differentiated cell types. Second, the Dab1 splice form initially found in retinal progenitor cells, Dab1-E, is no longer expressed in mature amacrine and ganglion cells. Restriction of Dab1-L to specific cell types indicates an alteration in the recognition of *Dab1* exons 7, 8 and 9B splice donor and recognition sequences by the splicing machinery. Changing the type, concentration and activity of splicing factors, SR proteins and hnRNPs, is often associated with the alternative recognition of splice sites (Stamm *et al.*, 2005). During retinal and brain development, Dab1 isoform switching is a highly coordinated process as two separate splice events must be simultaneously regulated: inclusion of exons 7 and 8 and exclusion of exon 9B. Expression of Dab1-L in amacrine and ganglion cells suggests the existence of retinal cell type-specific splicing factors or splicing activity.

It is interesting to postulate that the Dab1 transcriptional repression and isoform switching mechanisms which regulate Dab1 abundance and processing are interrelated. A number of studies show that splicing factors may associate with RNA polymerase II (Pol II), transcriptional co-activators/repressors, histone deacetylases and the chromatin of activated genes (Misteli and Spector, 1999; Gall *et al.*, 1999; Hirose and Manley, 2000; Dellaire *et al.*, 2002). This raises the intriguing possibility that splicing factors associated with formation of *Dab1-L* in amacrine and ganglion cells may positively regulate histone acetylation and/or Pol II-mediated transcription of *Dab1*. Likewise, the splicing factors associated with *Dab1-E* production in retinal progenitor cells may negatively regulate *Dab1* expression through similar but opposite mechanisms upon progenitor maturation into other cell types.

5.1.6 Dab1 may be a master adaptor and regulator of SFK-mediated signaling in neurons

A growing number of proteins and pathways are implicated with tyrosine phosphorylated Dab1 (Chapter 1, Figure 1.11). A commonality shared by many of these proteins is that they contain one or more SH2 domains which can associate with tyrosine phosphorylated Dab1 SFK or Abl/Crk motifs. Interestingly, these proteins can also undergo SFK-mediated tyrosine phosphorylation and are implicated in the modulation of cell migration and neuronal differentiation. The breadth of these interactions suggests that Dab1 may serve as a central scaffolding protein for cytoskeletal-regulating pathways to converge upon in a SFK-dependent manner. Therefore, we postulate that Dab1 serves dual adaptor functions. The first adaptor function would be to bridge the extracellular Reelin signal to activate intracellular SFKs. The second would be to bridge multiple SFK-dependent cytoskeletal signaling pathways. In this way Dab1 may act as a molecular sensor by which different Dab1 tyrosine phosphorylation events elicit different SFK-mediated cytoskeletal responses. To support this hypothesis, our mutation analysis of individual Dab1 tyrosine sites show that Dab1-mediated activation of SFKs can involve a combination of tyrosine phosphorylation sites, with the dual combinations of Y232/Y220 and Y232/Y285 resulting in increased SFK activation and neurite formation, while Y232 alone or the combination of Y185/Y220 results in an intermediate level of SFK activation and a more attenuated change in cell morphology.

Some preliminary data also suggest that Dab1-L can modulate SFK-dependent cellular responses subsequent to activation of SFKs. Cells transfected with Dab1-L display SFK activation and form numeous thin elongated processes. A close examination of these cell processes show that they have jagged bends, display some arborization and lack organization. We have recently obtained a Src kinase mammalian expression construct (pClcsrc) (kindly provided by Dr. D Fujita, U. Calgary) harboring a Y527F mutation (Src^{Y527F}). This mutation disrupts the inhibitory tyrosine phosphorylation of Src resulting in constitutive activation of this kinase (Hansen *et al.*, 1987; Cartwright *et al.*, 1987; Piwnica-Worms *et al.*, 1987). Primary chick retinal cells co-transfected with pClcsrc and pEGFP-C1 empty vector (as a fluorescent cell tracer) show abnormal cell morphology when compared to Dab1-L-expressing cells (data not shown). The GFP/Src^{Y527F}-expressing cells display two short and very thin processes that extend linearly from opposite ends of their cell bodies. Furthermore, there are far fewer GFP/Src^{Y527}-positive cells compared to GFP-expressing cells; however, we do observe a number of small, round cell-like structures which show GFP-aggregation and nuclear fragmentation, suggesting an induction of the apoptotic pathway in Src^{Y527F}-expressing cells. Co-transfection of pClcsrc with pEGFP-Dab1-L results in an increase in the number of GFP-positive cells and shows aspects of both Src^{Y527F}- and Dab1-L-like morphology (data not shown). These results are unexpected as we would have predicted that cellular expression of activated Src would have yielded similar results to those observed in cells expressing Dab1-L. These morphological differences between cells expressing Dab1-L and cells expressing activated Src suggest that Dab1-L can modulate downstream functions or responses in addition to those associated with activated SFKs.

5.1.7 The Dab1-Notch connection

A biochemical association between Disabled and Notch has been observed in *Drosophila* (Giniger, 1998). Although this study has failed to garner attention in the mammalian Dab1 field, the Disabled-Notch association may prove to be important in the context of retinal development. Like ApoER2 and VLDLR, Notch is a transmembrane receptor which contains cytosolic C-terminal NPxY docking sites. Notch has two primary roles in retinal development: (i) in ensuring the proper accumulation of retinal progenitor cells and (ii) in specification of the Müller glial cell lineage (Ohtsuka *et al.*, 1999; Furukawa *et al.*, 2000; Davis and Turner, 2001). We hypothesize that Dab1 isoform switching in the retina plays a role in Notch signaling. As both Notch and Dab1-E are expressed in retinal progenitor cells, we postulate that their interaction allows transduction of signals that promote neurogenesis. Upon retinal differentiation, Notch signaling is shut off in cells that are destined to remain of neuronal lineage. Similar to how Dab1 isoform switching (to Dab-L) recouples Reelin-mediated SFK activation, we propose that Dab1 isoform switching in maturing retinal cells may decouple Notch signaling thereby allowing their differentiation along a neuronal lineage.

5.2 FUTURE DIRECTIONS

5.2.1 Dab1 expression in primary chick retina cells

DNA transfection of our primary chick retinal cultures provides us with a unique opportunity to study the biochemical and physiological consequences of manipulating gene expression in neuronal cells. Others typically have used non-neuronal cells such as HEK 293T for these types of studies (Ballif *et al.*, 2004; Chen *et al.*, 2004; Huang *et al.*, 2004). However, the disadvantage of non-neuronal systems is the requirement to first generate a functional Reelin-Dab1 pathway through transfection of individual signaling components prior to studying the consequences of manipulating this pathway. Although the use of such cells allows for good transfection efficiency, the data gained from these analyses are strictly biochemical and do not provide a proper neuronal context. In addition to biochemical data, our culture system provides valuable morphological and cell behavior data in the context of neuronal development.

We will expand the use of our primary chick retinal cultures to study other aspects of Reelin-Dab1 signaling in the context of Dab1-E expression versus Dab1-L expression. So far, we have characterized changes in phosphotyrosine levels, activation of SFK and cell morphology as a function of Dab1 isoform expression. As a next step, we could characterize the effect that Dab1-E and Dab1-L have on neuronal migration. Using 2-D time-lapse microscopy and computational analysis, GFP-Dab1 positive retinal cells and their cellular processes could be tracked and measured as a function of time. These studies will provide insight as to the role of Dab1 isoforms on retinal cell movement and positioning during development. Furthermore, tracking the behavior of Dab1-L positive processes will provide data regarding Dab1-dependent neuritogenesis. Our analysis could be further expanded to include the various Dab1 tyrosine mutants described in Chapter 4. These studies will identify which Dab1 tyrosine sites are implicated in Reelin-dependent neuronal movement.

There are a number of postulated effectors of Dab1 signaling, including proteins that interact with the Dab1 SFK and Abl/Crk recognition motifs (as outlined in Chapter 1). Through western blot analysis and co-immunoprecipitation, we can use our Dab1-transfected retinal cells to identify differences in Dab1-protein interactions and/or the activation of these pathways in the context of Reelin/Dab1-E versus Reelin/Dab1-L signaling.

5.2.2 Dab1 expression in ovo

Our Dab1 transfection analyses have provided initial data describing the effects that Dab1 splice forms have on Reelin-Dab1 signaling and neuronal differentiation. In order to study these effects in the context of the natural retinal environment, it would be beneficial to use an *in vivo* model of development. As we have experience and success using the embryonic chick retina, our lab has begun studies using replication competent avian-specific RCAS/RCAN retroviral vectors to alter Dab1, Dab1-E and Dab1-L expression levels *in ovo*. Using this system, the eyes of chick embryos are micro-injected with GFP-Dab1 constructs and electroporated. For Dab1-E studies, embryos will be incubated until a time when Dab1-L is normally the predominant splice form (such as ED10), while

Dab-L studies will entail a shorter incubation period (ED5). Using antibodies against phosphotyrosine, phosphoSFK, GAP-43 and other retinal differentiation markers, GFP-Dab1-E or GFP-Dab1-L-positive retinas will be examined for changes associated with Reelin-mediated signaling and for differences in neuronal cell type and morphology. We predict that misexpression of Dab1-E at later stages of chick retinal development may adversely affect retinal differentiation and possibly reduce amacrine and ganglion cell formation. Similarly, we predict that Dab1-L missexpression in the early chick retina will result in premature neuronal differentiation. In addition, a similar analysis using isoform-specific siRNAs could be performed to assess the endogenous functions of Dab1 isoforms during retinal development. The use of an *in ovo* approach to misexpress or inhibit Dab1 splice forms will provide valuable insight as to the functions of Dab1-E and Dab1-L in the context of normal retinal development *in vivo*.

5.2.3 Mouse models of Dab1 alternative splicing

Another effective way to decipher the developmental function of these Dab1 isoforms will be to create *Dab1-E* and *Dab1-L* transgenic mice in a Dab1-null background (*scrambler* or *yotari*). By expressing Dab1-E, we may rescue Dab1 functions associated with neural progenitor cells while Dab1-L expression will rescue Dab1 functions associated with neuronal differentiation. Analyses of these transgenic mice should reveal differences in retinal architecture and neuronal traits attributed to differences in Reelin-dependent signaling and expression of Dab1-E and Dab1-L isoforms.

A mouse transgenic approach would also be effective in identifying the physiological contribution of individual Dab1 tyrosines (Y185, Y198, Y220 and Y232). So far, only one line of mice harboring Dab1 tyrosine mutations has been documented, *Dab1*^{5F} (Howell *et al.*, 2000). However, this mouse harbors mutations in all five tyrosines spanning Y185 to Y232. Although this mouse was effective in establishing the need for Dab1 tyrosine phosphorylation to relay the Reelin signal intracellularly, it is irrelevant in the context of studying individual Dab1 tyrosines and their involvement in this signaling pathway. Using a similar approach to generate Dab1 tyrosine→phenylalanine mutants (described in Chapter 4), we could produce Dab1 transgenic mice harboring multiple single, double and triple Y→F mutations. Comparison of the phenotypes displayed by these mice will help to establish a physiological role for individual Dab1 tyrosines in Dab1 activity, Reelin-Dab1 signaling and neuronal development. Furthermore, retinal tissue sections derived from these mutants could be used to study SFK activation, retinal cell morphology and activity of downstream effectors. Similarly, retinal protein extracts could be used to identify tyrosine- and phosphotyrosine-specific interactions and pathways implicated in Dab1 and Reelin-Dab1 signaling, respectively.

The inclusion of exon 9B, a domain with unknown function, in Dab1-E is intriguing as it provides evidence for a functional role for Dab1-E expression in neural/retinal progenitor cells. One way to identify a physiological role for exon

9B would be to generate *Dab1-E* transgenic lines that cannot "splice in" exon 9B. This could be achieved by altering the splice acceptor sequence at the start of exon 9B so that this exon is not included in the mature *Dab1-E* transcript. Any developmental differences detected between the retinas of exon 9B-modified and -unmodified *Dab1-E* transgenics would be attributed to the function of this exon and would provide insight as to its role in neural/retinal progenitor cells.

To address the asynchronous nature of the Dab1 isoform switching in neurons, the use of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) expression cassettes would allow us to track changes in the types of Dab1 isoforms expressed by different populations of neurons. We could generate a mouse gene targeting construct containing a *Dab1* genomic fragment spanning introns 6 to 10. We could place a CFP cassette in front of exon 7 with particular attention being placed on preserving *cis*-regulatory elements and splice donor and acceptor sequences associated with this exon. Similarly, a YFP cassette could be placed in front of exon 9B with the same emphasis placed on preserving similar elements. Analysis of neurons derived from resulting embryos expressing fluorescent Dab1 protein would provide us with an easy way to track isoform switching in all Dab1-expressing CNS cell types and would provide a convenient molecular marker to differentiate between neural progenitor cells (expressing YFP-Dab1-E) and neurons (expressing CFP-Dab1-L). Ultimately, this approach would allow us to track which Dab1 isoforms are expressed in each cell type at different stages of neuronal migration. In addition, we could correlate Dab1 isoform switching with neuronal lamination during retina and brain development.

5.2.4 Identification of cis-regulatory elements and trans-acting factors responsible for Dab1 isoform switching

Using a small antisense RNA approach (Vacek *et al.*, 2003), we could identify RNA elements responsible for splicing of *Dab1*. To identify splicing elements implicated in alternative splicing of the β-globin gene, Gorman and colleagues have previously developed the U7SmOPT vector which utilizes modified U7 RNAs for RNA-mediated redirection of alternative splicing. These vectors allow cloning of short double-stranded oligos (9 mers) that, upon cell transfection, will undergo U7-promoted RNA transcription and generate corresponding short antisense RNAs (Gorman *et al.*, 1998). RNAs found to bind specific *cis*-elements will act as competitors for *trans*-acting factors thereby altering splice site recognition and splicing of the corresponding exon.

We have previously found that prolonged culturing of our primary chick retinal cells results in endogenous Dab1 isoform switching (data not shown). Therefore, these cells are an ideal model system for studying alternative exon usage in *Dab1*. We will generate sequential sets of short double-stranded oligonucleotides that span a region flanking the splice acceptor sites and splice donor sites of either Dab1 exons 7 and 8 or exon 9B. These will be cloned into the U7SmOPT vector and each construct will be transfected into our primary chick retinal cells. Total RNA from these cultures will be isolated and analyzed by RT-PCR using the P1/P2 (for exon 7/8) or P3/P4 (for exon 9B) primer sets. Antisense RNAs that result in inclusion of these exons will indicate the presence of suppressor elements while RNAs that result in exon exclusion will indicate the presence of splice enhancer elements. Once we identify the region(s) containing these *cis*-elements, their sequences would be searched with splicing factor matrices (Liu *et al.*, 1998) or the ASD regulatory database (http://www.ebi.ac.uk/asd-srv/regseq.cgi) to identify the corresponding splicing factors (Thanaraj *et al.*, 2004). Once these splicing factors are identified, we would study their levels of expression (by western blot analysis) and their localization (by fluorescence immunolocalization) in chick retinal tissue derived from different embryonic stages. Our goal would be to identify any variations in expression within particular retinal cell types and correlate these results with Dab1 expression. Should we find no differences, we would then determine whether these factors undergo any form of post-translation modification, such as phosphorylation. The identification of a cell type- or developmental stage-specific splicing factor or splicing activity would play a major role in furthering our understanding of the mechanisms underlying Dab1 splicing and Reelin-Dab1 signaling. Furthermore, this would have important implications in the field of RNA splicing as few examples of such phenomena have been documented.

5.2.5 Additional Dab1 biochemical interactions

In Chapter 4, we have identified a number of novel Dab1-protein interactions. These data further implicate Dab1 in pathways related to actin-mediated transport, microtubule-mediated transport and protein degradation. These interactions will need to be confirmed and characterized using a variety of biochemical assays (co-immunoprecipitation, GST-pulldown analysis, interaction-domain mapping) and microscopy techniques (co-localization studies).

The association of Dab1 with SHP-2 is especially intriguing as we provide evidence of an additional mechanism by which Reelin/Dab1-L-mediated activation of SFKs is regulated. We postulate that tyrosine phosphorylated Dab1 (at Y198) mediates the association with SHP-2. To determine whether a phosphotyrosine-dependent interaction exists between Dab1 and SHP-2, we will need to use of a variety of biochemical techniques. We have recently obtained GST-fusion constructs containing the SHP-2 SH2 domain (kindly provided by Dr. D. Burshtyn, U. Alberta). Using this construct, we could perform GST-pulldown analysis using protein extracts derived from Dab1-L-transfected retinal cells. As our previous results indicate, we would expect GFP-Dab1-L protein to be phosphorylated at tyrosine-198 thereby ensuring the required phosphomodification to facilitate Dab1-L interaction with SHP-2. Because of the low transfection efficiency in these cells, we may have some difficulty pulling down adequate amounts of tyrosine phosphorylated Dab1. Therefore, we could use a cell line with a higher transfection efficiency, such as 293T. In order to force Dab1 phoshorylation at tyrosine-198, Dab1-L-expressing 293T cells would be pre-treated with pervanadate, a potent inhibitor of phosphatase activity. As a control, the Dab1-L^{Y198F} mutant would be used to determine the specificity of the Dab1-SHP-2 interaction. As stated in Chapter 4, we predict that the Dab1-SHP-2 interaction prevents an association between Dab1-L and SFKs thereby preventing SFK activation. To test this hypothesis, we could perform an *in vivo* competition experiment. For this, we would need to generate a mammalian expression construct of the SHP-2 SH2 domain and co-express increasing amounts of this domain with Dab1-L in primary chick retinal cells. The SH2 domain of SHP-2 does not possess any catalytic activity, therefore if the mechanism underlying suppression of Dab1-L-mediated SFK activation is through competitive inhibition by SHP-2, we expect to find decreasing amounts of SFK activation as we increase SHP-2 SH2 domain expression levels. Should the mechanism involve SHP-2 phosphatase activity, expression of the SHP-2 SH2 domain would have no effect and we would expect to see robust SFK activation.

Recent data indicate that phosphorylated Dab1 (tyrosine-198) associates with PLCγ1. Similarly, PLCγ1 undergoes tyrosine phosphorylation and activation by SFKs (Liao *et al.*, 1993; Marrero *et al.*, 1995). Activated PLCγ1 has been shown to modulate actin polymerization and cell motility (Piccolo *et al.*, 2002). Interestingly, the combined activities of PLCγ1 and SFKs lead to phosphorylation and activation of Abl kinase. It would be informative to examine the phosphorylation status of PLCγ1 in our Dab1-L-transfected retina cells using an antibody against phosphorylated PLCγ1 (commercially available). Should we find that PLCγ1 is indeed activated through Reelin-dependent Dab1/SFK signaling, further analysis would be carried out to identify how the Dab1/SFK/PLCγ1 pathway might regulate Abl/Crk-mediated cell migration and differentiation. Confirmation of this link may have profound implications in our understanding of the Reelin-Dab1 pathway.

5.3 SIGNIFICANCE

Our discovery of Dab1 isoform switching during neuronal development represents a novel mechanism in the regulation of signal transduction pathways. Our results point to a new way of modulating signaling in pathways that utilize cytoplasmic adaptor proteins to transduce external stimuli. Our example of developmentally-regulated alternative splicing, involving key SFK recognition motifs in Dab1, highlights a previously unknown innate ability of cells to decouple the pathways responsible for transduction of inappropriately-timed external signals. These cells retain the ability to become responsive to their environment through splicing-mediated inclusion of the appropriate stimuli-responsive domains that are required to recouple the signaling pathway. As alternative splicing is a widespread event and a number of cytoplasmic adaptor proteins exist, we predict that this mechanism is widely used to regulate a number of signal transduction pathways.

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